IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Cuttitta et al.
Application No. 09/931,700
Filed: August 16, 2001
Confirmation No. 3744
For: FUNCTIONAL ROLE OF ADRENOMEDULLIN (AM) AND THE GENE RELATED PRODUCT (PAMP) IN HUMAN PATHOLOGY AND PHYSIOLOGY
Examiner: Susan Ungar, Ph.D.
Art Unit: 1642
Attorney Reference No. 4239-63842-01

CERTIFICATE OF MAILING

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DECLARATION OF FRANK CUTTITTA, PH.D. UNDER § 1.132

1. I, Frank Cuttitta, Ph.D., am a co-inventor of the above-referenced patent application. I am currently a Principal Investigator at the Cell and Cancer Biology Branch of the National Cancer Institute, National Institutes of Health, and have been doing scientific research for approximately 30 years. I have been involved in research related to cancer biology for much of my career. My Curriculum Vitae is attached as **Exhibit A**.

2. The following experiment was performed in my laboratory, and shows that MoAbG6 inhibits lung cancer growth *in vivo* in a mouse lung cancer xenograft model (A549, human lung [adenocarcinoma] cancer cell line). The cancer cell line was obtained from the American Type Culture Collection. Tumor cells (1×10^7 in 50 µl of sterile 1 x PBS) were injected s.c. into the hind flank of female BALB/c mice. Groups of mice were randomized into treatment groups (10 mice per group) and were given MoAbG6 (100 µg i.p./mouse, three times per week) over a 28 day period. Tumor growth was monitored every 3-4 days by microcaliper measurements (HxWxL) and resulting volume determined (plotted as mm^3).

As shown in **Exhibit B**, at five and six weeks following tumor cell injection, MoAbG6-treated mice had a statistically significant smaller tumor volume than control mice (* = $p<0.05$). Thus, MoAbG6 inhibits human lung adenocarcinoma growth *in vivo*.

3. The following experiment was performed in my laboratory, and shows that MoAbG6 blocks adrenomedullin function and reduces both the number and size of metastases in an *in vivo* model of renal carcinoma.

The antitumor efficacy of MoAbG6 was evaluated in an orthotopic murine renal carcinoma (RENCA) metastasis model in syngeneic BALB/c mice essentially as described for the evaluation of other antiangiogenic agents (Wood *et al., Cancer Res.* 60:2178-2189, 2000; Drevs *et al, Cancer Res.* 60:4819-4824, 2000; Morita *et al., Br. J. Urol.* 4:416-421, 1994). For antitumor efficacy studies, RENCA cells (1×10^5 in 50 µl of sterile 1 x PBS) were injected into the renal subcapsular space of the left kidney of female BALB/c mice. Two days after recovery from surgical implantation, groups of mice were randomized into treatment groups and were given MoAbG6 (100 µg i.p./mouse, three times per week) over a 21- to 26-day period.

Mice were necropsied and lungs were harvested after RENCA challenge, fixed in formalin, paraffin embedded, sectioned (5 microns), and stained with hematoxylin and eosin. The metastatic lesions were counted and sized per slide and averaged for five slides.

The results of this experiment are shown in **Exhibit C**, panels A-D. Panels A and B show histological sections of lung tissue from control animals (panel A) and animals that received MoAbG6 (panel B). Panel C is a graph showing the number of metastatic lesions in control and MoAb-treated mice. Panel D is a graph showing the nodule size of metastatic lesions in control and MoAbG6-treated mice. The data is a representative example of results from 20 mice. Statistical analyses of tumor and lung weights were done by the Mann-Whitney rank-sum test, with $P < 0.05$ deemed significant. The MoAbG6-treated mice showed fewer metastases and smaller metastatic lesions than control mice, indicating that MoAbG6 (which blocks adrenomedullin function) reduces both the number and size of metastases *in vivo*.

4. **Exhibit D** (Ouafik *et al.*, *American Journal of Pathology* 160(4):1279-1292, 2002) is attached hereto. The reference is a published article from a peer-reviewed journal, and it demonstrates that an anti-human adrenomedullin antibody suppresses human glioblastoma

xenograft growth *in vivo*. Intratumoral injection of the anti-AM antibody reduced tumor size as compared to control antibody or saline (**Exhibit D**, Figure 9.)

5. **Exhibit E** (Martínez *et al.*, *Peptides* 20:1471-1478, 1999) is attached hereto. The reference is a published article from a peer-reviewed journal, and it demonstrates that MoAbG6 injected intraperitoneally produced significantly lower glucose responses in obese rats *in vivo* (see Figure 4). Thus, systemically (i.p.) administered anti-adrenomedullin antibody (MoAbG6) is not bound up by serum to the extent that it is rendered therapeutically ineffective, but is available to exert its effects on a variety of tissues, including pancreas.

6. **Exhibit F** (Cuttitta *et al.*, Nature 316(6031):823-826, 1985) is attached hereto. The reference is a published article from a peer-reviewed journal, and it describes how administration of an anti-bombesin monoclonal antibody at a dose of 200 μg per mouse i.p., three times per week, inhibits growth of human small-cell lung cancer in a mouse xenograft model (see Figure 2). Like adrenomedullin, bombesin is a peptide hormone, and the anti-bombesin monoclonal antibody disclosed in this reference has a binding affinity for bombesin that is similar to the binding affinity of MoAbG6 for adrenomedullin (approximately 10^{-9} M).

Based on the teachings in Cuttitta *et al.* (Nature 316(6031):823-826, 1985), a person skilled in the art who knew that 100 μg/ml of MoAbG6 inhibits human lung cancer cell growth *in vitro* would have expected that administration of about 200 μg per mouse, three times per week, for example about 100 to 300 μg of MoAbG6, would be successful at inhibiting adrenomedullin activity *in vivo*, and therefore would successfully inhibit human cancer growth *in vivo*, for example human lung adenocarcinoma growth.

7. The dose of MoAbG6 used in **Exhibits B** and **C** (100 μg i.p. per mouse) is equivalent to an *in vitro* concentration of approximately 5 μg/ml (100 μg per ~20g mouse). This is the lowest concentration of MoAbG6 shown in Figure 16A of the present application, which demonstrates that *in vitro* application of 5 μg/ml to100 μg/ml of MoAbG6 reduces growth of human lung cancer cells (MCF-7 cells). A person of skill in the art would have known to use a dose of MoAbG6 *in vivo* (for example, 100 μg per mouse) to achieve a tissue concentration of

MoAbG6 successful in inhibiting lung cancer cell growth *in vitro* (for example, 5 μg/ml – 100 μg/ml of MoAbG6).

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Frank Cuttitta, Ph.D. Date 10/11/04

Curriculum Vitae

Name: Frank Cuttitta

Date of Birth: November 7, 1947; Brooklyn, New York

Citizenship: United States

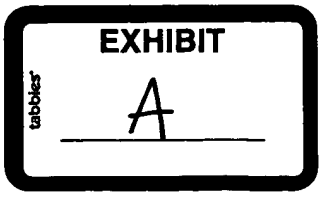
Marital Status: Married, 1973, two children

Education:

June 1965	Graduated Wheaton High School Wheaton, Maryland
June 1970	B.A. (Microbiology/Biochemistry), University of Maryland, College Park, Maryland
June 1980	Ph.D. (Microbiology/Immunology), University of Maryland, College Park, Maryland

Brief Chronology of Employment:

1970-1972	Microbiologist GS-5, Platelet Aggregation Studies, Dr. Sherly Johnson, V.A. Hospital, Washington, D.C.
1972-1975	Microbiologist GS-7, Thyroid Research, Dr. Louis Olner, V.A. Hospital, Washington, D.C.
1975-1978	Microbiologist GS-9, Sickle Cell Research, Drs. Geraldine Schechter/Paul McCurdy, V.A. Hospital, Washington, D.C.
1978-1980	Microbiologist GS-11, Monoclonal Antibody Development, Dr. John Minna, V.A. Hospital, Washington, D.C.
1980-1982	NIH Postdoctoral Fellowship, Dr. John Minna, V.A. Hospital, Washington, D.C.
1982-1984	Staff Fellow, NIH, NCI, DCT, Navy Medical Oncology Branch, NNMC, Bethesda, Maryland



1984-1986	Senior Staff Fellow, NIH, NCI, DCT, Navy Medical Oncology Branch, NNMC, Bethesda, Maryland
1986-1989	Research Assistant Professor of Medicine USUHS detailed to NCI-Navy Medical Oncolgy Branch, NNMC, Bethesda, Maryland
1989-1991	Research Associate Professor of Medicine USUHS detailed to NCI-Navy Medical Oncolgy Branch, NNMC, Bethesda, Maryland
1991-1995	Deputy Branch Chief, NIH, NCI, DCS, Biomarkers and Prevention Research Branch, Rockville, Maryland.
1995-1996	Acting Branch Chief, NIH, NCI, DCS, Biomarkers and Prevention Research Branch, Rockville, Maryland.
1997-present	Senior Investigator, NIH, NCI, CCR, Cell and Cancer Biology Branch, Chief, Cancer Cell Peptide Regulator Section Bethesda, Maryland.

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Postdoctoral Fellows

Steve Rosen, M.D. (Northwestern University)	1980-1981
James Mulshine, M.D. (Holy Cross University)	1981-1983
Sylvia Fargion, M.D. (Tumor Institute of Milano - Italy)	1981-1983

Austin Doyle, M.D. (University of Maryland)	1983-1984
Philip Kasprzyk, Ph.D. (Pennsylvania State University)	1985-1989
Kathryn A. Quinn, Ph.D. (University of Queensland - Australia)	1991-1994
Theodore Elsasser, Ph.D. (USDA - sabbatical)	1995-1997
Luis Montuenga, Ph.D. (University of Navarra - Spain)	1995-1998
Alfredo Martínez, Ph.D. (University of Navarra - Spain)	1994-present
Mercedes Garayoa, Ph.D. (University of Navarra - Spain)	1997-1999
Rubén Pío, Ph.D. (University of Navarra - Spain)	1998-2000
Enrique Zudaire, Ph.D. (University of Navarra – Spain)	2000-present
Elizabeth Warner, M.D. (Department of Surgery, Georgetown University)	2002-2004
Sergio Portal, Ph.D. (University of Navarra – Spain)	2003-present

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Blair High School Magnet Program Chethan Gangireddy	1995 (summer)
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Stone Ridge Country Day School Christine Piringer	1996 (summer)
Georgetown Prep High School Brian Henderson	1997 (summer)

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Book Chapters

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Filing Date - September 9, 1985
First Revision of Patent - June 20, 1989
Second Revision of Patent - September 10, 1990
Final Patent Awarded - February 13, 1991

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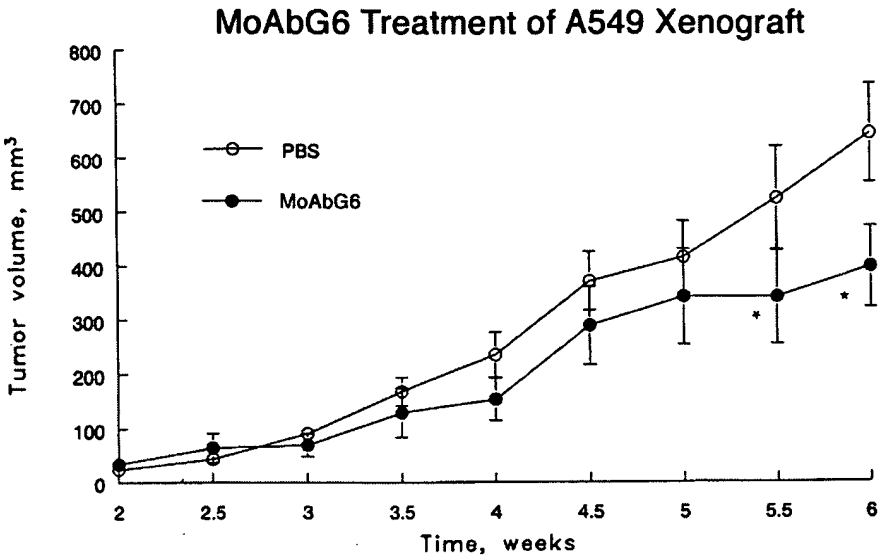
U.S Provisional Patent Application No. 60/500,650
Filing Date – September 8, 2003

Confidential Disclosure Agreements (CDA)/Material Transfer Agreements (MTA)/Cooperative Research and Development Agreements (CRADA) with US Biomedical/Pharmaceutical Companies:

Abgenix, Inc., Fremont, CA – CDA/MTA established in 2002 and the initiation of a CRADA started in 2003 for the company to develop humanized neutralizing monoclonal antibodies to AM/PAMP for potential use in clinical trails as intervention drugs against human cancers.

Panorama Research, Inc., Mountain View, CA – CDA established in 2003 and the initiation of a CRADA starting in 2003 for the company to develop alternative peptide antagonists or small molecule inhibitors to PAMP as potential intervention drugs against human cancers.



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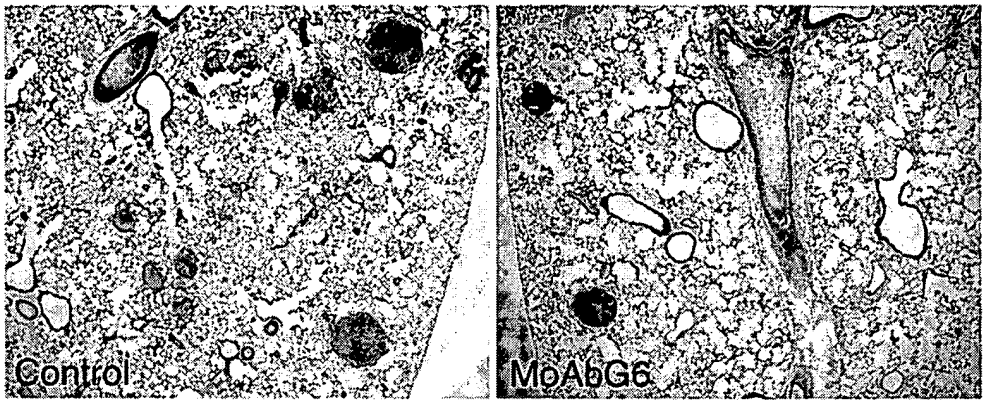

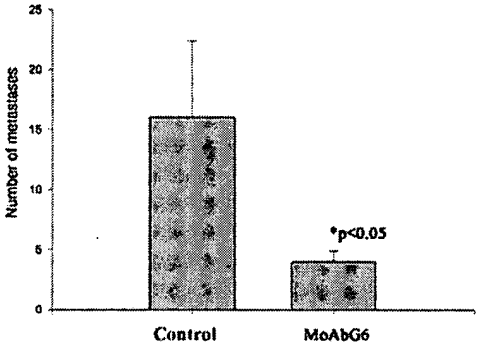

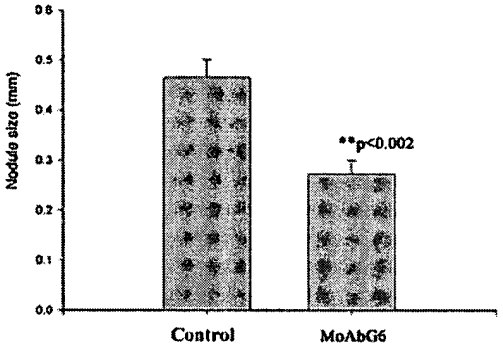

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Neutralization of Adrenomedullin Inhibits the Growth of Human Glioblastoma Cell Lines *in Vitro* and Suppresses Tumor Xenograft Growth *in Vivo*

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Presently, there is no effective treatment for glioblastoma, the most malignant and common brain tumor. Growth factors are potential targets for therapeutic strategies because they are essential for tumor growth and progression. Peptidylglycine α-amidating monooxygenase is the enzyme producing α-amidated bioactive peptides from their inactive glycine-extended precursors. The high expression of peptidylglycine α-amidating monooxygenase mRNA in glioblastoma and glioma cell lines points to the involvement of α-amidated peptides in tumorigenic growth processes in the brain. After screening of amidated peptides, it was found that human glioblastoma cell lines express high levels of adrenomedullin (AM) mRNA, and that immunoreactive AM is released into the culture medium. AM is a multifunctional regulatory peptide with mitogenic and angiogenic capabilities among others. Real-time quantitative reverse transcriptase-polymerase chain reaction analysis showed that AM mRNA was correlated to the tumor type and grade, with high expression in all glioblastomas analyzed, whereas a low expression was found in anaplastic astrocytomas and barely detectable levels in low-grade astrocytomas and oligodendrogliomas. In the present study we also demonstrate the presence of mRNA encoding the putative AM receptors, calcitonin receptor-like receptor/receptor activity-modifying protein-2 and -3 (CRLR/RAMP2; CRLR/RAMP3) in both glioma tissues and glioblastoma cell lines and further show that exogenously added AM can stimulate the growth of these glioblastoma cells *in vitro*. These findings suggest that AM may function as an autocrine growth factor for glioblastoma cells. One way to test the autocrine hypothesis is to interrupt the function of the endogenously produced AM. Herein, we demonstrate that a polyclonal antibody specific to AM, blocks the binding of the hormone to its cellular receptors and decreases by 33% ($P < 0.001$) the growth of U87 glioblastoma cells *in vitro*. Intratumoral administration of the anti-AM antibody resulted in a 70% ($P < 0.001$) reduction in subcutaneous U87 xenograft weight 21 days after treatment. Furthermore, the density of vessels was decreased in the antibody-treated tumors. These findings support that AM may function as a potent autocrine/paracrine growth factor for human glioblastomas and demonstrate that inhibition of the action of AM (produced by tumor cells) may suppress tumor growth *in vivo*. *(Am J Pathol 2002, 160:1279–1292)*

Malignant glioblastomas are highly aggressive tumors with a median patient survival time of 9 to 14 months.[1] They are characterized by rapidly dividing cells, invasion into normal brain, and a high degree of vascularity. The histological grade of malignant gliomas, based on vascular proliferation, endothelial cell hyperplasia, and microvessel count, has been shown to be inversely related to prognosis.[2] Growth factors regulate cell proliferation and differentiation and are directly involved in neoplastic transformation.[3] A critical feature of many peptides (hormones, growth factors, neuropeptides) that enables them to mediate intracellular communication, is a carboxyl-terminal α-amide group.[4,5] A single enzyme complex peptidylglycine α-amidating monooxygenase (PAM; EC1.14.17.3) is responsible for the α-amidation of these peptides and hormones.[5] PAM activity has been found in a number of endocrine tumors (secreting α-amidated

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peptides), such as medullary thyroid carcinoma, pheochromocytoma,[6] pancreatic tumors (secreting vasoactive intestinal peptide),[7] and human pituitary tumors.[8,9] The finding that PAM immunoreactivity was also detected in several unexpected cell types, such as ependyma, choroid plexus, oligodendroglia, and Schwann cells[10] prompted us to investigate whether PAM expression could be detected in gliomas.

The presence of PAM in gliomas should suggest the active synthesis of α-amidated peptides functioning as growth factors and playing a role in the regulation of both cell proliferation and differentiation. To better define the role of PAM in human gliomas, we have sought α-amidated factors involved in tissue growth. A preliminary screening of amidated peptides present in these tumors and in derived cell lines have demonstrated that the messenger ribonucleic acid for preproadrenomedullin is by far the most predominant message encoding for two α-amidated peptides, namely proadrenomedullin N-terminal 20 peptide and adrenomedullin.

AM shows some homology with calcitonin gene-related peptide (CGRP), and has therefore been added to the calcitonin/CGRP/amylin peptide family.[11] Subsequent work revealed that AM is produced by a wide variety of tissues, most notably the adrenal medulla, lung, kidney, and heart atrium.[12] It has been shown to mediate a multifunctional response in cell culture and animal systems and these responses include growth regulation and induction of angiogenesis.[13–16] Further studies have demonstrated AM expression in a variety of human tumors of both pulmonary and neural lineage including small cell lung cancer, lung adenocarcinoma, bronchoalveolar carcinoma, squamous cell carcinoma of the lung and lung carcinoids, ganglioneuroblastoma, and neuroblastoma.[17,18] Several observations suggest that AM may be potentially involved in tumorigenesis.[13] A variety of transformed cell lines, including the glioblastoma cell lines T98G and A172[19,20] express the AM mRNA and secrete AM.

Three AM receptors with different affinities for AM [L1, RDC1, and calcitonin receptor-like receptor (CRLR)], have been cloned and sequenced.[21–23] All of them belong to the seven-transmembrane domain G protein-coupled receptor superfamily. Interestingly, CRLR requires the presence of modulating proteins with a single transmembrane domain known as receptor activity-modifying proteins (RAMPs).[24] RAMP1 presents CRLR at the plasma membrane as a terminally glycosylated, mature glycoprotein and a CGRP receptor, whereas RAMP2 and RAMP3 present CRLR as an immature, core glycosylated AM receptor.[24,25]

Although the expression of AM has been demonstrated to be up-regulated in several tumors,[17–19] the exact biological effects of AM in tumorigenesis remain obscure. Proof of the role of AM in tumor growth requires the demonstration that inhibition of AM action influences tumor growth *in vivo*. The availability of specific polyclonal antibodies capable of blocking AM interaction with its cell surface receptors allowed us to test the hypothesis directly.

In the present study we demonstrate: 1) the presence of the appropriate posttranslational processing enzymes (PAM) in gliomas and glioma cell lines suggesting the capacity of these cells to synthesize α-amidated peptide(s); 2) the expression of AM_{1-52} amide, and AM-R in glioma cells implicating a possible autocrine growth mechanism; 3) that AM acts as a growth factor for glioma cells *in vitro*; and 4) that treatment with a polyclonal antibody specific for AM decreases the proliferation of the glioma cells *in vitro*, and inhibits the growth of a human glioma xenograft *in vivo*.

Materials and Methods

Cell Culture

Human glioblastoma cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in minimum essential medium (U373, U138, and U87) or in L15 medium (SW1783 and SW1088) containing penicillin (50 U/ml), streptomycin (50 μg/ml), glutamine (1 mg/ml), and supplemented with 10% fetal bovine serum. Cells were cultured under a moist 5% CO_2/95% air atmosphere, and fed with fresh medium every 2 days, being routinely monitored for mycoplasma contamination (Roche Molecular Biochemicals, Meylan, France). Cells growing exponentially were harvested and prepared for RNA analysis and amidation activity measurement. All culture media components were purchased from Invitrogen Life Technologies (Paris, France).

Cell Proliferation Assay

The effects of AM_{1-52} amide, $CGRP_{8-37}$ amide, AM_{22-52} amide, and rabbit anti-human AM antibody (purified IgG) on cell proliferation was examined at the indicated time points by the MTT [3,(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay.[26,27] After 2, 4, 6, and 8 days growth at 37°C in a humidified 95% air-5% CO_2 atmosphere, the dye and solubilization solutions were added from the Promega proliferation assay (Promega, Lyon, France) which is a variation of the MTT assay.[26,27] The Bio-Tek Microplate Manager plate reader and software was used to determine the change in the number of viable cells from dye reduction measured by absorbance at 570 nm.

Patients and Tissue Preparation

Tumors tissues from patients operated on for glioma at the Department of Neurosurgery, CHU Timone, Marseille, France, were studied. Our series include according to the World Health Organization histopathological classification,[28] 4 oligodendrogliomas grade II (low grade), 7 anaplastic oligodendrogliomas, 4 grade II astrocytomas (low grade), 4 anaplastic astrocytomas, and 14 glioblastomas (grade IV). Brain tumor samples were collected at the time of surgery and immediately stored in liquid nitrogen until used for RNA extraction. Nontumoral telencephalon was obtained from a patient submitted to a surgical procedure for epilepsy treatment. All tissue procurement protocols were approved by the relevant institutional committees

(University of Aix-Marseille) and were undertaken under informed consent of each patient and participant.

Northern Blot Analysis

Total RNA was prepared from tumors tissues and cell lines using the acid guanidinium isothiocyanate/phenol/chloroform procedure.[29] Northern blot analysis was performed essentially as described previously.[30] Briefly, total RNA (20 μg) was resolved on 1% agarose-formaldehyde-denaturing gel. The denatured RNAs were transferred to Hybond-N membranes (Amersham-Pharmacia Biotech, Orsay, France) by capillary action in 10× standard saline citrate [1.5 mol/L NaCl, 0.15 mol/L sodium citrate (pH 7.0)], cross-linked by UV irradiation and hybridized to [α-^{32}P]-labeled human 1.1-kb PAM cDNA[31] and 1.2-kb AM cDNA,[32] respectively. Filters were prehybridized, hybridized, and washed as described previously.[30] To correct for differences in loading and/or transfer, blots were stripped and hybridized to cDNA probes derived from frog rRNA.[33] The autoradiograms were analyzed by measurement of absorbance by scanner-densitometer using NIH Image 1.54 software (National Institutes of Health, Bethesda, MD). The hybridization signals of PAM and AM mRNAs were normalized to that of 18S rRNA. The results were expressed as the ratio of PAM or AM mRNAs absorbances to 18S rRNA absorbance.

Preparation of Tissue Extracts and Amidation Assay

Cells were scraped from culture wells into ice-cold phosphate-buffered saline (PBS), collected, and prepared for amidation assay as described previously.[30]

Peptide Extraction and Radioimmunoassay

Cell pellets (6×10^6 cells) were boiled in 0.5 mol/L of acetic acid for 20 minutes (1:10, w/v). After homogenization with a Potter apparatus, cell suspensions were centrifuged at 24,000 × *g* for 15 minutes. The pellets were stored at −20°C until assayed for protein content using the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Interchim, Paris). The supernatant was lyophilized and the resulting residues were resuspended in radioimmunoassay (RIA) buffer.[12] The RIA of AM was performed as previously reported,[12] using the anti-serum against human $AM_{1\text{-}52}$ amide developed in our laboratory, and used at a final dilution of 1:30,000. To measure the immunoreactive AM (IR-AM) in the culture medium, the medium was extracted by the previously reported method[12] using Sep-PaK C18 cartridges (Waters, Milford, MA). Intra- and interassay coefficients of variation were 5% (n = 10) and 8% (n = 7), respectively.

Chromatographic characterization of IR-AM in the culture medium was performed by reverse-phase high performance liquid chromatography (HPLC) using a μBondapak C18 column (3.9 × 300 mm; Waters). The conditioned medium (80 ml) was extracted using Sep-PaK C18 cartridges. The extract was reconstituted with water containing 0.1% (v/v) trifluoroacetic acid and loaded onto the column. The HPLC analysis was performed with a linear gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid from 10 to 60% at a flow rate of 1 ml/min/fraction throughout 50 minutes. Each fraction (1 ml) was collected, dried, and assayed for IR-AM.

Western Blot Analysis

The medium of U87, U373, or SW1088 cultures was replaced with serum-free defined medium. After 24 hours of cell culture, this conditioned medium was harvested and concentrated 70-fold using a SpeedVac concentrator (Savant Instruments, Inc.). An equal quantity (30 μl) was prepared for electrophoresis by making them 2% in sodium dodecyl sulfate and 5% in 2-mercaptoethanol and heated to 95°C for 5 minutes. Samples of medium were fractionated on slab gels containing 12% acrylamide and 0.25% *N,N*-bis-acrylamide using the buffer system of Laemmli.[34] Proteins were electrophoretically transferred to Hybond-C membranes (Amersham Pharmacia Biotech) for 1 hour at 210 mA and visualized with Ponceau S (Sigma). Molecular weights were estimated by comparison with the prestained protein molecular weight standards (Invitrogen Life Technologies). Hybond-C strips were blocked in PBS buffer containing 5% nonfat dry milk and incubated overnight in 1:1000 dilution of rabbit anti-serum anti-human AM, and washed three times in PBS. Signals were revealed using an enhanced chemiluminescence kit (ECL kit, Amersham Pharmacia Biotech). Specificity control consisted of a duplicate membrane incubated in antigen-preabsorbed (10 nmol/ml anti-AM) anti-serum.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real-time quantitative PCR was used to accurately detect the changes of AM and GAPDH gene copies. The cycle at which the amplification plot crosses the threshold (CT) is known to accurately reflect relative mRNA values.[35,36] Total RNA (2 μg) DNA-free was reverse-transcribed into complementary DNA (cDNA) using 1 μg of hexamers (Pharmacia Biotech, Orsay, France) and M-MLV reverse transcriptase as described by the manufacturer (Invitrogen Life Technologies). Human AM and PAM and GAPDH mRNAs were amplified (AM: forward primer, 5′-TGCCCAGACCCTTATTCGG-3′ and reverse primer, 5′-AGTTGTTCATGCTCTGGCGG-3′; PAM: forward primer, 5′-CACTGATTGGACGGCAGAG-3′ and reverse primer, 5′-CATCACTAGACGTGCCACCA-3′; GAPDH: forward primer 5′-CAAATTCCATGGCACCGTC-3′ and reverse primer 5′-CCCATCTGATTTTGGAGGGA-3′), detected, and quantitated in real-time using the ABI Prism 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA) as described previously.[35,36]

The *Taq* Man probes for AM, PAM, and GAPDH were 5′-ACATGAAGGGTGCCTCTCGAAGCCC-3′; 5′-TTTTG-GTGACCTACTGGCTGCAA-3′ and 5′-CCCATCACCAT-

CTTCCAGGAGCGAG-3′, respectively. The amplification mixture contained cDNA derived from 50 to 150 ng of total RNA, 0.2 μmol/L of primer, and 0.1 μmol/L of *Taq* Man probe in 50 mmol/L of salt and 5 mmol/L of $MgCl_2$. A two-step PCR was performed for 35 cycles. Denaturation was done at 94°C for 20 seconds, and annealing/extension at 60°C for 30 seconds. The reaction produced a 115-bp PCR product for AM, one of 155 bp for PAM, and one of 101 bp for GAPDH. To determine the accuracy of the assay, total RNA was reverse-transcribed and amplified on 3 separate days. The interassay accuracy of amplification for the 3 days was 8%. For quantitation of the data, AM mRNA levels were normalized to the GAPDH mRNA levels in the same reaction. To create standard curves for each gene, RNAs were produced by *in vitro* transcription from linearized templates corresponding to AM, PAM, and GAPDH cDNA constructs using T_7 or T_3 polymerases and reverse-transcribed to cDNA.

Taq Man PCR Assay Conditions for AM, PAM, and GAPDH mRNAs

Using the fluorogenic probes for AM, PAM, and GAPDH with the experimental conditions defined above, we obtained a linear relationship between the RNA concentration (previously transcribed into cDNA) and the fluorescent signal (ΔRQ) for AM, PAM, and GAPDH RNAs in 1-to 250-pg DNA target. For each unknown sample, we determined the ΔRQ values for all three genes and the results were expressed as fg of AM or PAM per pg GAPDH.

RT-PCR of CRLR and RAMP mRNAs

Total RNA (5 μg) from human gliomas and glioma cell lines, was reverse-transcribed into cDNA using 1 μg of oligodT_{12-18} (Parmacia PL, Paris, France) as primer in a 20-μl reaction volume containing 50 mmol/L of Tris-HCl (pH 8.3), 75 mmol/L of KCl, 3 mmol/L of $MgCl_2$, and each of four dNTPs (Pharmacia), 20 U of Rnasin (Promega, Lyon) and 400 U of M-MLV reverse transcriptase (Invitrogen Life Technologies) at 37°C for 60 minutes. The primers used in the PCR were designed to be specific for CRLR, RAMP2, and RAMP3 and not to cross-hybridize with any other known sequences as reported by Martinez and colleagues.[37] Human CRLR, RAMP2, and RAMP3 were amplified (CRLR: forward primer 5′-GTAATGTTAACACCCACGAGAAAG-3′ and reverse primer 5′-ATCCCCAGCCAAGAAAATAATAC-3′; RAMP2: forward primer 5′-GGATATAGGCGCCCCCACAC-3′ and reverse primer 5′-GGAAGCCCAGGTCAAACAACTCT-3′; and RAMP 3: forward primer 5′-CGCAGCAAACGCACCGACAC-3′ and reverse primer 5′-GAGCCAGGGCAGGAACCAGAGATG-3′). PCRs were performed in a 50-μl volume, with 20 mmol/L of Tris-HCL (pH 7.4, 25°C), 50 mmol/L of KCl, 1.5 mmol/L of $MgCL_2$, 0.1% Triton X-100, 200 μmol/L each of four dNTPs, 1 μmol/L of each primer, cDNA derived from the equivalent of 300 ng of total RNA, and 2.5 U of Expand Long Template (Roche Molecular Biochemicals). Samples were subjected to 35 cycles in the MJ Research thermal cycler (MJ Research Inc., La Jolla, CA). Cycle parameters were generally as follows: the initial denaturation step was at 94°C for 4 minutes, the repeat cycle consisted of annealing at 50°C for CRLR, and 58°C for RAMP2 and RAMP3 for 40 seconds, followed by extension at 68°C for 50 seconds and denaturation at 94°C for 30 seconds; the last extension time was lengthened to 10 minutes. Samples were fractionated on a 1.2% agarose gels in 89 mmol/L of Tris, 89 mmol/L of borate, 2.5 mmol/L of ethylenediaminetetraacetic acid, pH 8.0, buffer. After staining with ethidium bromide, gels were photographed and prepared for Southern transfer by soaking for 15 minutes in 1.5 mol/L NaCl and 0.5 N NaOH and then for 30 minutes in 1 mol/L Tris-HCl, pH 8.0, and 1.5 mol/L NaCl and transferred to Hybond-N membrane as described.[30] The filters were hybridized with the internal probes for CRLR, RAMP2, and RAMP3, 5′-TGGGACATTTGCAACTAACAG-3′, 5′-GGGGACGGTGAAGAACTATGAGAC-3′, 5′-TCTAGGGCCAGTGGAGGAAAAT-3′, respectively. The filters were washed as previously described[30] and exposed to film to verify the identity of the bands.

Development of Anti-Human AM Antibody

The polyclonal antibody against human AM was developed by use of the synthetic peptide corresponding to the entire AM_{1-52} amide peptide (Bachem). Female New Zealand rabbits received injections at multiple subcutaneous sites with 120 μg of synthetic peptide emulsified with complete Freund's adjuvant. Then the rabbits were further immunized at 3-week intervals with 100 μg of AM_{1-52} amide emulsified with incomplete Freund's adjuvant.[38] The anti-sera obtained after the fourth booster injection were screened for anti-AM activity, and then affinity purified on rProtein A Sepharose Fast Flow columns (Amersham Pharmacia Biotech).

Binding of ^{125}I-Labeled AM

Glioblastoma cells were cultured in 24-well plates for 48 hours (15×10^4 cells/well) and then serum-starved for 24 hours. After washing with PBS, cells were incubated in TIS medium (MEM plus 10 μg/ml transferrin, 10 μg/ml insulin, and 3×10^{-8} mol/L sodium selenite) at 25°C for 120 minutes with the radioactive tracer in the presence or absence of an excess (10^{-6} mol/L) of unlabeled AM as previously described.[39] Iodination of synthetic hAM was performed by the chloramine T method,[40] and purified by reverse HPLC; monoiodinated [^{125}I] hAM (SA, 350 Ci/mmol) was used in the experiments. In binding inhibition studies, cells were incubated with tracer and increasing concentrations of the anti-AM antibody. At the end of the incubation period, cells were extensively washed with cold PBS containing 0.2% bovine serum albumin, solubilized with 0.2 mol/L of sodium hydroxide, and analyzed for bound radioactivity in a γ-spectrometer. Specific binding was obtained by subtracting nonspecific binding in the presence of excess unlabeled hAM from total bind-

ing. Data points represent the mean of three experiments, each of which was performed in triplicate.

Animal Studies

Animal work was performed in the animal facility of the school of medicine in accordance with institutional guidelines. Male 4-to-5-week-old athymic NMRI (*nu/nu*) mice (Janvier, Laval Le Genest, France) were used. Mice were acclimated and housed in sterile cages in groups of four or less under laminar flow hoods in a temperature-controlled room with a 12-hour light/12-hour dark schedule, and fed autoclaved chow and water *ad libitum.*

Treatment of Glioblastoma Tumor Xenografts

Athymic NMRI (*nu/nu*) nude mice were implanted with U87 glioblastoma cells. For the cell implantations, U87 cells, grown in culture, were washed with PBS, dispersed in a 0.05% solution of trypsin, and resuspended. After centrifugation (4000 rpm for 20 minutes at 8°C), the cell pellet was resuspended in PBS and the final concentration was adjusted to 3×10^7 cells/ml and the suspension was placed on ice. After the site was cleaned with ethanol, 0.1 ml (3×10^6 cells) of the suspension were subcutaneously injected in the right flanks of nude mice. Tumors were measured with a dial-caliper, and volumes were determined using the formula width × length × height × 0.52 (for ellipsoid form). After 12 days, when the primary tumors were 1350 to 1500 mm^3 in size, animals were randomly divided into three groups. One group (n = 20) received intratumoral injection of the anti-AM antibody (200 μg of purified IgG) as a suspension in PBS in a volume of 0.2 ml every 3 days. As control, one group (n = 7) received an irrelevant antibody (IgG of the same isotype) and the other group (n = 7) received comparable injections of the vehicle alone (PBS). Mice were sacrificed at the indicated time.

Immunohistochemical Analysis

Tumor specimens were embedded in Tissue-Tek and frozen on dry ice/butane, and stored at −80°C. Frozen sections (5 μm) were cut on a Leica cryostat. Sections of each specimen were stained using hematoxylin and eosin (H&E). Immunohistochemistry was performed using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). For the purpose of assessing tumor vascularity and proliferation characteristics, tissue sections were evaluated using antibodies to factor VIII-related antigen (von Willebrand factor) (DAKO, Denmark) and Ki-67 nuclear antigen (DAKO). To ensure that representative tumor tissue sections were evaluated, all tumors were sectioned through their largest diameter and then at least five thin slices were made from each half of the resected tumor. Detection was performed using a diaminobenzidine chromogen, which resulted in a positive brown staining. Sections were counterstained with hematoxylin, dehydrated in ethanol, and mounted with glass coverslips. Negative control slides were obtained by omitting the primary antibody. Ki-67 staining was quantified by counting the number of positively stained cells of all nuclei in 15 randomly chosen fields.

In situ detection of apoptosis was measured by the terminal deoxynucleotidyltransferase (Tdt)-mediated dUTP nick end-labeling (TUNEL) method using the apopTag Plus Kit (Intergen, Gaithersburg, MD) followed by counterstaining with 1% methyl green. Apoptosis was quantified by determining the percentage of positively stained cells for all nuclei in 20 randomly chosen fields per section at ×200 magnification.

Tumor Vascular Density

Quantitation of vessel count was performed by a procedure described by Wedner and colleagues.[41] The blood vessels were counted randomly from nonnecrotic areas in each tumor section in a ×200 microscope field (1.0 mm^2) (Olympus BH2, Tokyo, Japan), on vWF-stained tissue sections. Vascular density was defined by averaging the number of vessels with lumen in at least eight of the most vascular areas.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analyses were performed by using the one-way analysis of variance followed by Fisher's protected least significant difference test (Statview 512; Brain Power Inc., Calabasas, CA). The difference was considered significant if the P value was less than 0.05.

Results

Human Glioma and Glioma Cell Lines Express High Levels of PAM mRNA

Total RNA from surgically resected human glioma, human glioma cell lines, and nontumoral brain tissue was prepared to assess steady-state levels of PAM transcripts. Northern blot analysis revealed the presence of an ~4-kb PAM mRNA visualized using a 2.2-kb human PAM cDNA probe.[31] High levels of PAM transcript were expressed in human gliomas (Figure 1A) and in the glioma cell lines (Figure 1C) as compared to nontumoral tissue (Figure 1A). The PAM cDNA probe was removed from the blots, and the amount of ribosomal RNA present in each sample was determined by hybridization to a cDNA probe for ribosomal RNA (Figure 1, A and C). In glioma tumors, the amount of PAM mRNA, quantified by densitometry and normalized to 18S ribosomal RNA, seemed to correlate with the tumor grade. PAM mRNA levels were 9- to 15-fold higher in malignant glioblastomas and fourfold to sixfold higher in low-grade gliomas as compared to nontumoral brain tissue (Figure 1B). Most estab-

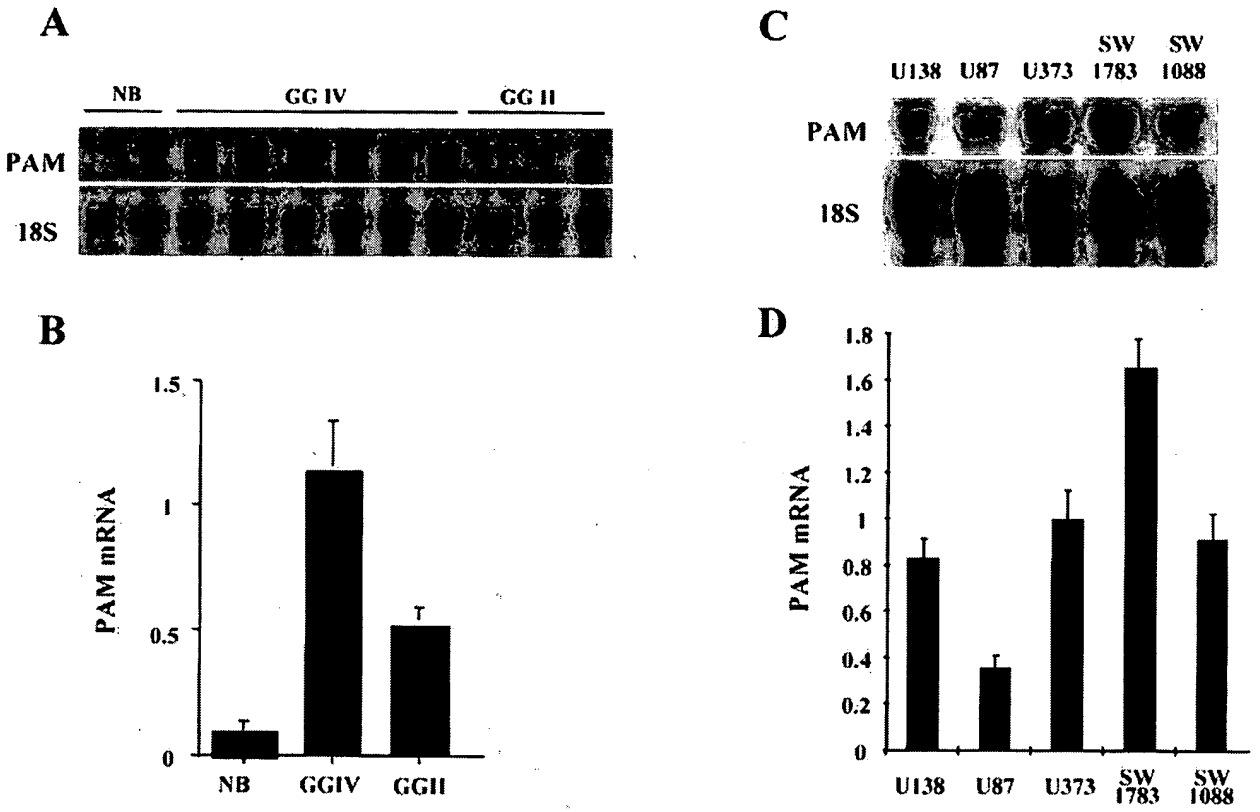

Figure 1. Representative Northern blot analysis showing the expression of PAM mRNA in human glioma, glioblastoma cell lines, and nontumoral brain tissue. **A** and **C:** Total RNA (20 μg) was fractionated on 1% agarose gel and transferred to Hybond-N membrane. The blots were hybridized with a 2.2-kb human PAM cDNA probe and exposed to X-ray film for 24 hours at −70°C with an intensifying screen. The blots were subsequently stripped and reprobed with a cDNA probe corresponding to 18S rRNA to permit correction for the amount of sample actually transferred to Hybond-N membrane. **B** and **D:** For densitometric analysis, the amount of PAM mRNA was normalized to the amount of 18S rRNA; this arbitrary ratio was used to express relative tissue and cell line PAM mRNA levels. Results shown for glioblastoma cell lines are the mean ± SEM of four independent experiments. NB, nontumoral brain; GGIV, glioma grade IV; GGII, glioma grade II.

lished cell lines derived from human glioblastoma constantly expressed high levels of PAM mRNA (Figure 1D).

PAM Activity in Glioma Cell Lines

The amidation activity of extracts of each cell line was assayed using α-*N*-acetyl-Tyr-Val-Gly substrate at pH 5.5.[30] Total PAM activity was calculated by summing the amount of PAM activity estimated in both particulate and soluble fractions (Figure 2). The glioblastoma cell lines U373 and SW1783 consistently displayed higher levels of PAM activity (80 ± 4 and 74 ± 4 pmol/mg protein/hour, respectively) than U87 (56 ± 4.6 pmol/mg protein/hour); U138 (57 ± 4.5 pmol/mg protein/hour) and SW1088 (54 ± 2.7 pmol/mg protein/hour) cell lines. Subsequent studies have indicated that the culture medium of these cell lines also displayed substantial amounts of secreted PAM (not shown). The amount of PAM activity in each cell line represents a balance between synthesis, storage, inactivation, and secretion of the enzyme. Therefore, the correspondence between glioma cell line PAM expression, as determined by Northern analysis (Figure 1D), and the corresponding cell line PAM activity level may not be quantitatively equal. The expression of PAM mRNA in both gliomas and glioma cell lines and the presence of PAM activity in these glioma cell lines demonstrate their capacity to produce α-amidated peptides.

AM mRNA Is Expressed in Glioma Cell Lines

To determine the substrate(s) for PAM in the glioma cell lines, we screened these cells by RT-PCR for the expression of many α-amidated peptides known to have mitogenic effects on tumor cells. A very high expression was found for AM in contrast to a weak expression of neuropeptide Y and growth hormone-releasing hormone, and no expression of calcitonin gene-related peptide (CGRP), cholecystokinin, and vasoactive intestinal peptide (not shown).

To assess the levels of AM mRNA in the glioma cell lines and human nontumoral telencephalon, total RNA was subjected to Northern blot analysis, and AM mRNA was visualized using a human AM radiolabeled cDNA.[32] The size of the messenger transcript was ~1.6 kb (Figure 3A), corresponding to what has been found in other AM-producing tissues.[42] The amount of AM mRNA was then normalized to the amount of 18S ribosomal RNA (Figure 3B). The Northern blot analysis demonstrated that all glioma cell lines express AM mRNA, whereas no expression of AM mRNA was detected in nontumoral telen-

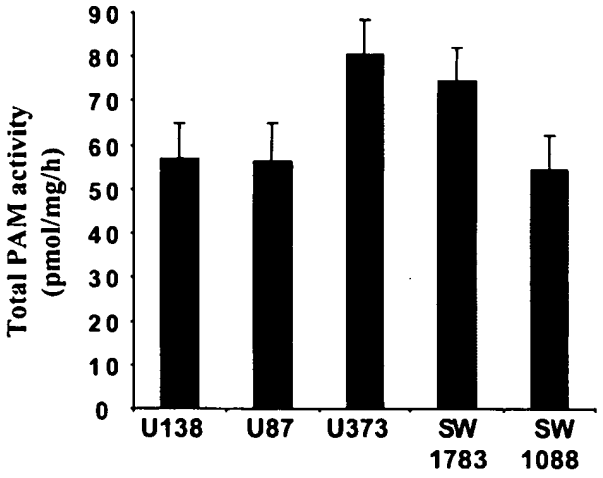

Figure 2. PAM activity in glioblastoma cell lines. Crude particulate and soluble fractions were prepared at least three times from each cell line. Assays for PAM activity were performed in duplicate with at least two different amounts of protein. Total PAM-specific activity was calculated by taking into account the amount of protein in the two fractions. The mean total specific activities ± SEM are plotted.

cephalon tissue. However, the expression of AM mRNA in nontumoral brain tissue was observed by RT-PCR (unpublished data),[32] suggesting a low level of expression of AM mRNA in nontumoral human brain tissue. These results confirm previously reported data in T98G and A172 glioblastoma cell lines.[19,20]

Production and Secretion of AM Peptide by Human Glioma Cell Lines

To investigate whether glioma cells could produce the AM protein, RIA, reverse phase-HPLC, and Western blot analysis were performed. Immunoreactive AM was detected in both the cell extracts and culture media. All of the glioma cell lines produced and secreted IR-AM. The amount of IR-AM produced and secreted by different cell lines is shown in Table 1. Reverse-phase HPLC showed that IR-AM in the medium of U87 cells contained a single peak eluting in the position of AM_{1-52} amide (Figure 4A). The conditioned medium of U87 cells also contained two minor peaks eluting earlier (Figure 4A), which could be either a material very similar to AM_{1-52} amide, probably an AM precursor fragment, or AM with some minor modifications. Western blot analysis of the conditioned medium obtained from U87, U373, and SW1088 cells demonstrated an immunoreactive band of 6 kd that presumably represents the authentic peptide (Figure 4B). In addition, immunoblot analysis of HPLC fraction containing IR-AM revealed a major 6-kd immunoreactive band (Figure 4B). The specificity of our immune-detection assay was confirmed by an antibody absorption control that eliminated the specific bands (Figure 4C). Taken together, these findings confirmed that cultured glioma cells themselves produce and secrete AM peptide.

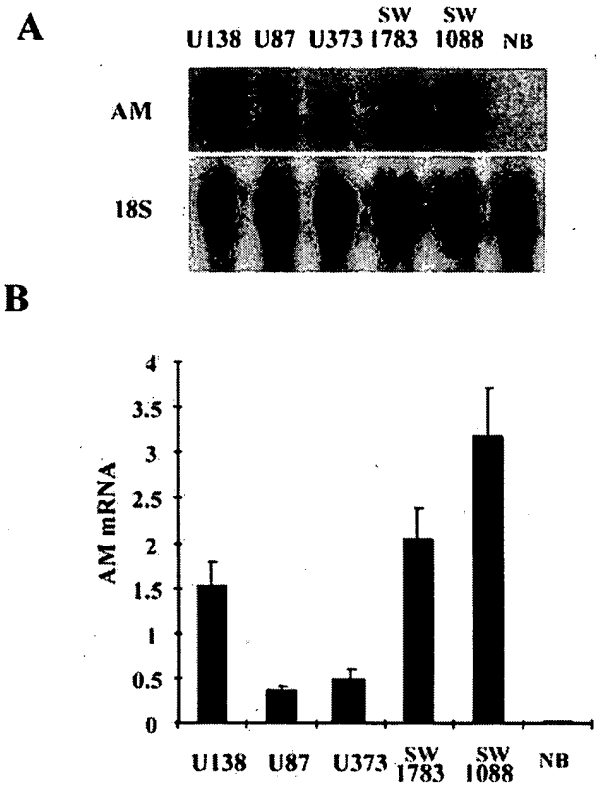

Figure 3. Expression of AM mRNA in glioblastoma cell lines and nontumoral brain tissue. **A:** Total RNA (15 μg) prepared from glioblastoma cell lines and brain tissue was subjected to Northern blot analysis. The blot was hybridized with human AM cDNA probe. The membrane was subsequently stripped and hybridized with 18S ribosomal cDNA. **B:** The autoradiograms were densitometrically analyzed and the amount of AM mRNA was normalized to the amount of 18S rRNA. Data are presented as mean ± SEM of four independent experiments.

Expression of AM and PAM mRNAs in Human Glioma

Total RNA from human gliomas was prepared to assess steady-state levels of AM and PAM mRNA transcripts. Real-time quantitative RT-PCR analysis was performed on tumor fragments of the 31 gliomas. Based on World Health Organization histopathological classification, the present series included 4 low-grade oligodendrogliomas (grade II), 7 anaplastic oligodendrogliomas, 4 low-grade astrocytomas (grade II), 4 anaplastic astrocytomas, and 14 high-grade gliomas (grade IV). Quantification of PAM mRNA transcripts revealed higher PAM mRNA levels in glioblastomas compared with low-grade and anaplastic astrocytomas. The mean level of PAM mRNA expression

Table 1. AM Content in Cell Extracts and Culture Medium in Glioma Cell Lines

	Cellular content, fmol/mg protein	Culture medium content, fmol/ml/h
U87	2.312 ± 0.256	0.415 ± 0.014
U138	2.564 ± 0.288	0.248 ± 0.044
SW1088	5.398 ± 0.127	0.488 ± 0.011
U373	2.583 ± 0.250	0.231 ± 0.012
SW1783	9.892 ± 0.101	0.104 ± 0.013

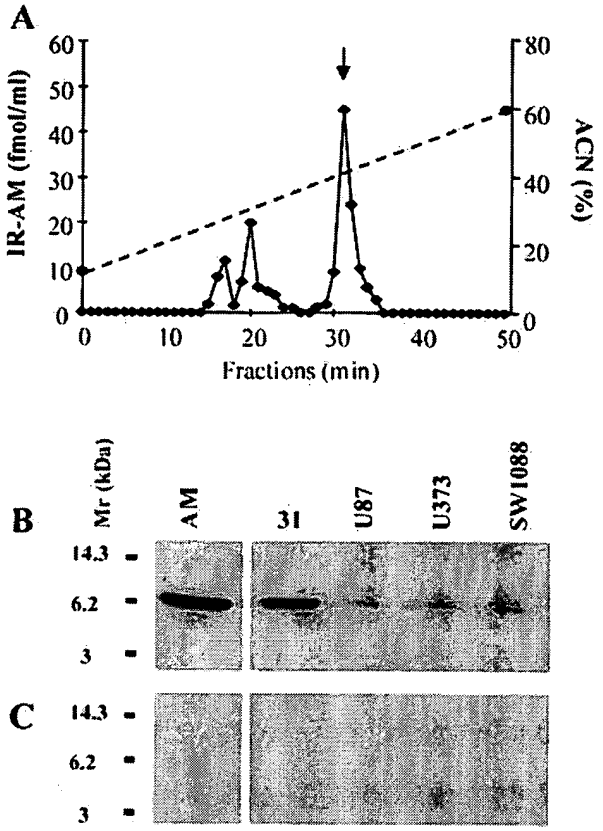

Figure 4. Reverse-phase HPLC profile and Western blot analysis of glioblastoma cell-conditioned media. **A:** Fractionation of 100 ml of U87 (concentrated to 1 ml before injection) compared with the elution time of synthetic AM at 31 minutes (**arrow**). **B:** After the media of subconfluent U87, SW1088, and U373 cells were changed, the media 24 hours later were collected and concentrated 50-fold. Western blot analysis of these media using anti-human AM antibody showed a single immunoreactive band. The reverse phase-HPLC fraction at 31 minutes contains the 6-kd entity. The amount of synthetic AM is 10 ng. **C:** These immunoreactive bands disappeared when we used the preabsorbed anti-AM antibody.

was 748 ± 87 fg/pg GAPDH mRNA in glioblastomas, whereas it was 262 ± 73 fg/pg and 189 ± 49 fg/pg for anaplastic and low-grade astrocytomas, respectively. Quantification of AM mRNA transcripts revealed a high level of AM mRNA in glioblastomas, compared to low-grade and anaplastic astrocytomas and oligodendrogliomas (Figure 5). The individual patterns of AM mRNA expression are presented in Figure 5. The mean level of AM mRNA expression was 55.8 ± 14.3 fg/pg GAPDH mRNA in glioblastomas, whereas it was 13.1 ± 3.2 fg/pg and 2.06 ± 0.8 fg/pg for anaplastic and low-grade astrocytomas, respectively. Among the glioblastomas, the individual pattern of expression was highly variable; in particular, five tumors (samples 1, 3, 5, 11, and 14) expressed AM mRNA levels >70 fg/pg GAPDH mRNA. Very low to null expression was detected in anaplastic oligodendrogliomas (4.12 ± 0.53 fg/pg) and low-grade oligodendrogliomas (0.34 ± 0.2 fg/pg). Omission of the reverse transcriptase eliminated the signal, which indicated that it was not attributable to contaminating genomic DNA (not shown).

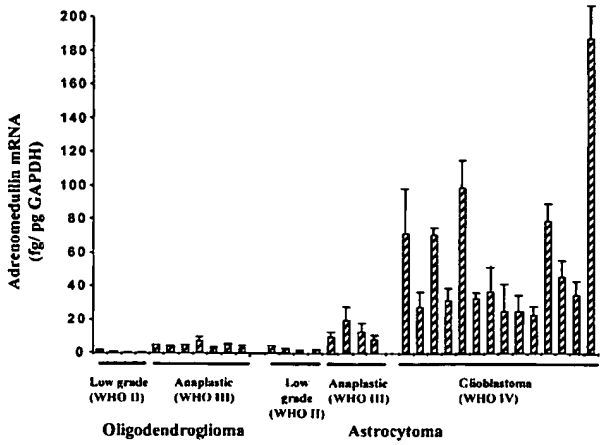

Figure 5. Real-time quantitative RT-PCR analysis of AM mRNA levels in glioma. Total DNA-free RNA from the oligodendroglioma (low grade and anaplastic), astrocytoma (low grade and anaplastic), and glioblastoma were transcribed to cDNA and subjected to quantitative RT-PCR using the ABI Prism 7700 sequence detection system for the estimation of relative AM to GAPDH mRNA ratio as described in Materials and Methods. Each column with bar shows mean ± SD (n = 3).

RAMP and CRLR mRNAs Are Expressed in Gliomas and Glioma Cell Lines

To determine whether the human brain gliomas and glioma cell lines express CRLR, RAMP2, and RAMP3, we examined the expression of their mRNA by RT-PCR. Subsequent Southern blot analysis of the RT-PCR products with the corresponding probes demonstrated in most of the gliomas and the cell lines bands of the expected sizes corresponding to the mRNA encoding CRLR as well as RAMP2 and RAMP3 (Figure 6). No bands are seen in the controls omitting reverse transcriptase enzyme (not shown). Examination of the expression of L1 and RDC1 receptors by RT-PCR showed no expression of these two types of receptors in the glioma cell lines (unpublished data).

Characterization of Anti-Human AM Antibody

The anti-AM polyclonal antibody (purified IgG) showed very low cross-reactivity with AM-related peptides such as AM_{22-52} amide, AM_{26-52} amide, and AM_{13-37}. Calcitonin, $CGRP_{1-37}$ amide, $CGRP_{8-37}$ amide, and amylin showed insignificant anti-AM antibody binding despite some homology with AM (Table 2). In addition, no cross-

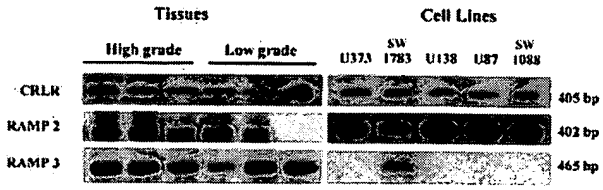

Figure 6. Expression of RAMP and CRLR mRNAs. Total RNA from high- and low-grade gliomas and glioblastoma cell lines was subjected to RT-PCR using the three pairs of primers and PCR conditions were as described in Materials and Methods. The PCR products were fractionated on agarose gels, prepared for Southern blot analysis, and hybridized with the corresponding probe. The number of bp present in PCR products are indicated on the **right**.

Table 2. Cross-Reactivity of the Anti-Adrenomedullin (Human) Antibody with AM-Related Peptides

Specificity	
AM (human, 1–52)	100%
AM (human, 22–52)	10%
AM (human, 26–52)	9%
AM (human, 13–37)	1%
CGRP (human, 1–37)	<0.1%
CGRP (human, 8–37)	<0.1%
Amylin	0%
Sensitivity	
IC_{50} 0.06 pmol/ml (antiserum dilution: ×30,000)	

reactivities against the following peptides were observed: [ACTH (1-24), endothelin-1, AVP, CRF, TRH, substance P, and ANF]. The Western blot analysis clearly supports the antibody's specificity for AM (Figure 4, B and C). We next tested the ability of the antibody to block the binding of labeled ^{125}I-AM to its cell-surface receptor on U87 cells (Figure 7). Anti-AM antibody blocked AM-receptor interaction in a dose-related manner (Figure 7). An indifferent rabbit anti-TRH antibody, of the same isotype as the anti-AM antibody (IgG) at 20 μg, did not significantly affect AM-receptor interaction (<1% inhibition).

Effect of AM and Anti-AM Antibody on the Proliferation of U87 Cells in Vitro

It is now clear from a vast number of studies that the major effect on AM-stimulated cells is an elevation of cAMP,[13] a signal transduction pathway known to modulate cellular growth.[43] To investigate this aspect, we used the MTT assay technique to examine the effects of AM on the growth of glioma cell lines. U87 cells cultured in serum-free medium were exposed to 2×10^{-7} mol/L of AM for the indicated time, and the effect on the proliferation was followed by the MTT assay. As shown in Figure 8, AM at 2×10^{-7} mol/L stimulated the proliferation of U87 by 13% ($P < 0.02$) and 12% ($P < 0.02$) after 6 and 8 days of treatment, respectively.

To test the autocrine hypothesis, a polyclonal anti-AM antibody (purified IgG) was examined for its effect on the

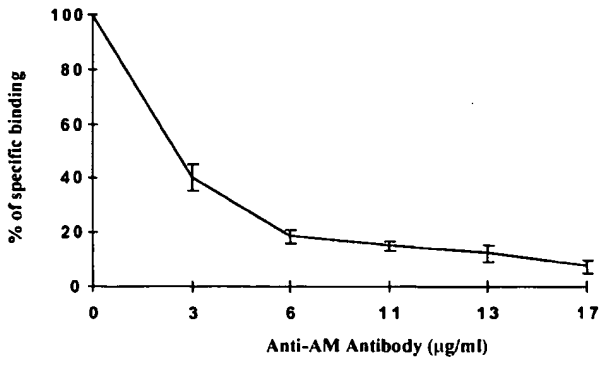

Figure 7. Concentration-dependent inhibition of ^{125}I-labeled AM binding by anti-AM antibody. Glioblastoma cells (U87) were incubated for 120 minutes at 25°C in the presence of ^{125}I-labeled AM (60 $\mu ci/\mu g^{-1}$, 7×10^4 cpm per test) and increasing concentrations of anti-AM antibody. Binding is expressed as a percentage of specific bound radioligand; each point is the mean of data from three independent experiments.

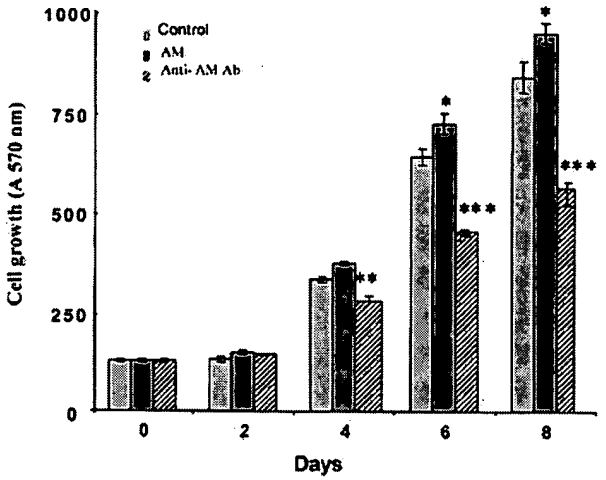

Figure 8. Effect of AM and anti-AM antibody on the growth of U87 cells *in vitro*. For proliferation assays, tumor cells were seeded at the density of 2×10^3 per well in 12 multiwell plates in the presence of TIS medium (MEM plus 10 μg/ml transferrin, 10 μg/ml insulin, and 3×10^{-8} sodium selenite) or MEM supplemented with 0.5% FBS (previously treated at 58°C for 1 hour), 2 mmol/L of glutamine, and antibiotics. AM at 2×10^{-7} mol/L and anti-AM antibody (20 μg/ml) were added. As controls, a boiled anti-AM antibody and a control IgG of irrelevant specificity were used (not shown). For each treatment, six wells were prepared for MTT assays or exposed to trypsin and cells were counted in a Coulter counter (not shown). **Vertical bars** represent SEM of seven independent experiments. *, $P < 0.05$; ***, $P < 0.0001$.

in vitro growth of a glioblastoma cell line. U87 cells cultured *in vitro* in serum-free medium were exposed to the anti-AM antibody at 20 μg/ml and the effect on proliferation was assessed with the MTT assay. The inhibition of proliferation reached 16% ($P < 0.01$), 28% ($P < 0.001$), and 33% ($P < 0.001$) by 4, 6, and 8 days of treatment, respectively, when compared to controls (Figure 8). In contrast, 20 μg/ml of the IgG of irrelevant specificity or boiled anti-AM antibody showed no inhibition of cell growth in the same assay (not shown). Furthermore, we could demonstrate specificity by reversing the effects of the anti-AM antibody, with the addition of synthetic AM at 1 μmol/L (not shown). Taken together, these data strongly suggest that AM acts as an autocrine regulator of glioma cell proliferation. To determine whether there is a relationship between growth suppression by anti-AM antibody and the level of tumor cell AM expression, the analysis of SW1088 cell line that expresses higher levels of AM than U87 cell line was undertaken. After 6 and 8 days of treatment, anti-AM antibody at 20 μg/ml inhibited the proliferation of SW1088 cells by 16% ($P < 0.002$) and 23% ($P < 0.0001$), respectively, as compared to controls. Interestingly, the U373 cell line that secretes less AM than U87 and SW1088 cell lines showed 40% ($P < 0.001$) inhibition of proliferation after 8 days of anti-AM antibody treatment.

Inhibitory Effect of hAM(22-52)-NH_2 on hAM-Mediated Cell Growth

To confirm that endogenous hAM produced by the glioblastoma cell lines acts as an autocrine growth factor, U87 glioma cells were incubated for up to 8 days in the absence or presence of the peptide antagonists AM_{22-52}

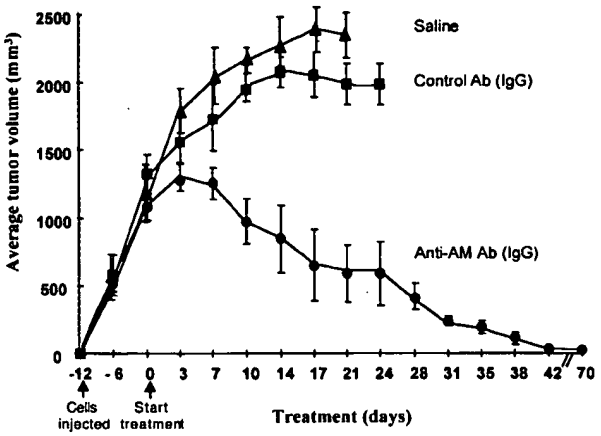

Figure 9. The effect of anti-AM antibody on tumor growth *in vivo*. The human glioblastoma U87 (3×10^6 cells), was subcutaneously implanted in *nu/nu* mice. Mice bearing U87 tumors were treated with anti-AM antibody, control IgG of irrelevant specificity, or saline vehicle. The sizes of the glioblastoma xenografts were determined by measuring the volume of the tumors.

amide at 10^{-6} mol/L and $CGRP_{8-37}$ amide at 10^{-6} mol/L, respectively. The growth of these cells showed a slight but significant decrease 13% ($P < 0.001$) in the presence of AM_{22-52} amide as compared to control cells. However, no decrease in the basal growth rate of the cells treated with $CGRP_{8-37}$ amide could be observed. These results support that AM acts as an autocrine regulator of glioma cell proliferation through a specific functional receptor present in these cells such as the CRLR/RAMP2 complex.

Effects of Anti-AM Antibody on Subcutaneous Established Human Glioma Xenografts

Mice were inoculated subcutaneous in the right flank with U87 cells. The tumors reached a size of 1350 ± 170 mm^3, 12 days after inoculation. Figure 9 shows the tumor growth curves of three groups of mice bearing U87 glioma xenografts, that were treated every 3 days with the anti-AM antibody, a control IgG of irrelevant specificity, or saline vehicle, respectively. Treatment was administered by intratumoral injection and tumor growth was monitored as a function of tumor volume throughout the time of therapy. The growth of U87 glioma xenografts was significantly inhibited by the anti-AM antibody when compared to both control groups. Twenty-one days after treatment, a group of animals were sacrificed, and tumor size and vascularity were assessed. The control gliomas were large, red, and hypervascular, whereas anti-AM antibody-treated gliomas were small and white with few visible surface blood vessels. The mean tumor volumes in the controls and in the anti-AM antibody group were 2150 mm^3 and 646 mm^3, respectively, at 21 days after treatment (Figure 9), and the mean tumor weights were 4.05 and 1 g, respectively. Intratumoral injections of anti-AM antibody for 7 weeks resulted in a further suppression of tumor growth (tumor mass in all treated mice < 40 mm^3) (Figure 9). The mean tumor volume of treated tumor at the end of experiment at day 70 was 2% of the initial pretreatment mean tumor volume.

Immunohistochemistry

Tumors treated with the anti-AM antibody had less vascularization than tumors treated with control IgG or saline, as seen by H&E staining (Figure 10A). This phenomenon was confirmed by von Willebrand factor (vWF) immunohistochemistry (Figure 10A). In addition, the anti-AM antibody-treated tumors were significantly less vascular than the control tumors ($P < 0.0001$; Figure 10, A and B). The mean vessel area was significantly higher in the control tumors as compared to the anti-AM antibody-treated tumors ($P < 0.0001$; Figure 10, A and B). However, there was no significant difference in FVIII-related antigen-stained cells in the anti-AM antibody-treated tumors and the control tumors as determined by direct counting (Figure 10, A and B). Accordingly and despite the fact that the apoptosis labeling is heterogeneous among the tumors, the apoptotic index of the anti-AM antibody-treated tumors was ~fourfold to fivefold higher than the control tumors ($P < 0.0005$; Figure 10C) and proliferative indices measured after staining for Ki-67 nuclear antigen revealed a significant decrease in tumors treated with the anti-AM antibody when compared to controls ($P < 0.016$; Figure 10C).

Discussion

Malignant gliomas are the most common primary tumors of the central nervous system.[1] At the present time the prognosis for patients with glioblastoma is poor. A better understanding of the cellular mechanisms underlying the growth and progression of glioma[1,44] may lead to improved therapy. For example, identifying factors produced in the tumor and elucidating their roles in tumor development may provide clues for improving therapy. However, greater benefit may be gained by identifying control points in the pathways of the synthesis of growth factors. In the present study, we showed an increased expression of PAM mRNA in gliomas. Levels of PAM mRNA were substantially elevated in malignant grade IV glioblastomas (9- to 15-fold) and grade II astrocytomas (4- to sixfold) compared to nontumoral brain tissue. As shown by the enzyme assays, PAM is active in all of the human glioblastoma cell lines, which indicates the capacity to α-amidate products if the appropriate prepropeptides, endoproteases, and exoproteases as well as reducing equivalents, are available. The increase in PAM biosynthesis is associated with the production of α-amidated peptides,[45] some of which could act as autocrine/paracrine factors and influence the response of neighboring neoplastic cells. A previous study on small cell lung cancer[46] has shown that bombesin/GRP, an α-amidated peptide, acts as an autocrine growth factor in these cells[47] which further supports a key role of PAM in tumor growth. A preliminary survey of amidated peptides present in glioblastoma-derived cell lines (U373, U138, U87, SW1783, and SW1088) demonstrated that AM is predominately represented both as mRNA and immunoreactive peptide in these cell lines. Similar data have

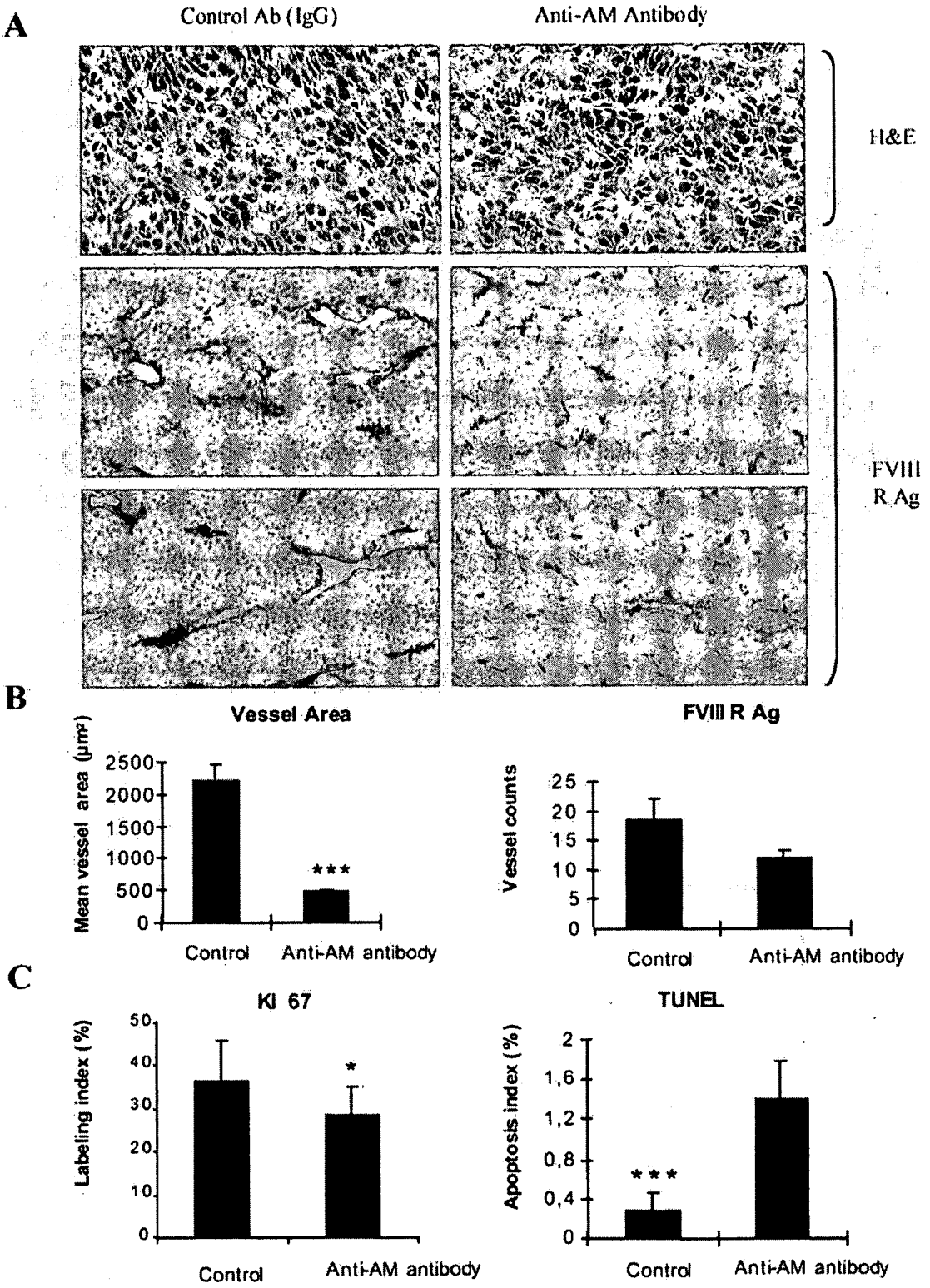

Figure 10. Effect of the anti-AM antibody on a human glioma xenograft. U87 cells (3×10^6) were subcutaneously implanted into athymic (*nu/nu*) mice. After 12 days, when the primary tumors were 1350 to 1500 mm^3 in size, animals were randomly divided into three groups and treated as described in Materials and Methods. Twenty-one days later, animals were sacrificed, and the subcutaneous glioma tumors were harvested. **A:** Microphotographs of immunohistochemical-stained tumor sections for H&E, and FVIII antigen (*FVIII RAg*), in control and anti-AM antibody-treated tumors. **B:** Quantitative assessment of the cell density that stained positive for FVIII-related antigen (*FVIII RAg*), and the mean vessel area expressed in μm^2 was measured through a microscope using a NIH image 1.62 Software for analysis ($n = 5$; values represent the mean; bars, SD; ***, $P < 0.0001$). **C:** The percentage of cells in active cell cycle or undergoing apoptosis was determined by staining for Ki-67 nuclear antigen or TUNEL assay (see Materials and Methods), respectively ($n = 5$; values represent the mean; bars, SD *, $P < 0.016$; ***, $P < 0.0005$).

been reported for the T98G and A172 glioblastoma cell lines.[19,20]

The expression of AM mRNA in 31 samples of human glioma was assessed by a real-time quantitative RT-PCR. The data demonstrated that a high expression of AM was restricted to the most aggressive form of glioma, namely glioblastoma whereas it was low in anaplastic astrocytoma and barely detectable in the low-grade astrocytoma and oligodendroglioma (Figure 5). Recently, an 11-fold overexpression of AM in one pool of five primary glioblastomas has been reported by Lal and colleagues[48] as determined by serial analysis gene expression (SAGE). Similar data were reported by Takahashi and colleagues.[19] The finding of an increased PAM expression in the lower grade tumors with no overexpression of AM at this time may suggest the possibility of synthesis of α-amidated peptide(s) other than those investigated in the present work.

The correlation of AM expression to the grade of glioma support the hypothesis that AM may participate in the progression of gliomas. Glioblastomas (World Health Organization glioma grade IV) are characterized not only by a nuclear atypia and a high mitotic rate, but also by a high vascular proliferation and necrotic foci (presumably hypoxic regions).[49] Recently, Garayoa and colleagues[50] demonstrated that the expression of AM mRNA in a variety of human cell lines is highly induced by hypoxia. Kitamuro and colleagues[51] confirmed the induction by hypoxia of AM mRNA in T98G. Recently, we demonstrated that reduced oxygen tension (1% O_2) or exposure to hypoxia mimetics such as desferrioxamine mesylate (DFX) or $CoCl_2$, induced AM mRNA expression in U87 cells (unpublished data). These results suggest that the resultant reduction in tissue oxygen tension may lead to an increased expression of AM mRNA in glioblastoma.

Previous studies have demonstrated the ability of reduced oxygen tension to mediate elevations in AM message/protein expression in several animals and cell systems. In this sense, hypoxia was shown to induce AM gene expression and secretion in cultured human umbilical vein endothelial cells;[52] focal ischemic regions of the rat brain show high AM mRNA expression,[53] and patients with chronic obstructive pulmonary disease have elevated AM plasma levels.[54]

AM binding has been demonstrated in most cell types and human tissues.[13] Sone and colleagues[55] reported that specific binding sites for AM were present in every region of human brain. Herein, we demonstrate the expression of AM receptor (CRLR and RAMP2 and RAMP3) in the tumors as well as in glioma cell lines. We further demonstrate that ^{125}I-AM binds to these cells. Moody and colleagues[39] demonstrated that a rat glioma cells line (C6) could bind human AM at high affinity (kd = 24 nmol/L), had an approximate receptor density of 36,000 sites per cell, and exogenous AM could augment intracellular cAMP/c-fos expression.

The presence of both AM and AM-R opens up for the possibility of AM being an autocrine/paracrine growth factor in gliomas. This hypothesis is supported by our work showing that AM stimulates U87, SW1088, and U373 cell growth. Although being significant, the stimulation was not strikingly high most probably because of the saturation of the receptors by the endogenous produced AM that may obscure the effects of external added AM.

Proliferation assays revealed that cell growth of U87 cells could be significantly suppressed by a neutralizing anti-AM antibody that blocks the binding of AM to cells, and this inhibition could be reversed by the addition of the exogenous AM (not shown); thus, the anti-AM antibody produced growth inhibition *in vitro* was most likely the result of a blocking of the autocrine/paracrine effects of AM produced by the glioma cells. These results thus suggest that AM may function as an autocrine growth factor involved in the growth control of glioblastoma cells such as U87, U373, and SW1088. Although $AM_{22\text{-}52}$ amide is not an especially potent antagonist, the slight decrease in cell growth is in agreement with the data described above. This set of characteristics, together with the binding assay experiments, clearly implicates the existence of an autocrine loop mechanism that could potentially drive neoplastic growth as has been described for other peptides.[47,56] The lack of complete inhibition *in vitro* may be explained by the effect of additional growth factors produced by the tumor cells such as fibroblast growth factors, epidermal growth factor, platelet-derived growth factor, and transforming growth factor-β.[57,58] In further support of a functional role of AM in glioblastoma cells is the recent report by Moody and colleagues,[39] who reported that monoclonal antibody C6, which neutralizes AM, significantly inhibited the rat glioma cell line C6 proliferation *in vitro* and decreased the ability of AM to elevate *c-fos* mRNA. In addition, Miller and colleagues[20] reported that a neutralizing anti-AM monoclonal antibody was growth inhibitory *in vitro* to H157 (adenosquamous), H720 (lung carcinoid), MCF-7 (breast adenocarcinoma), and OVCAR-3 (ovarian adenocarcinoma) cells, which also showed both ^{125}I-AM binding and AM-stimulated cAMP.

To extend the *in vitro* observations, *in vivo* experiments were performed. Our results demonstrated that the anti-AM antibody could be efficiently delivered *in vivo* and significantly suppress the growth of established glioblastoma xenografts. This effect was observed when treatment was initiated 12 days after implantation at a time when tumors had reached a significant volume. After 1 week of treatment, tumors in mice treated with control IgG, or saline grew progressively to a size that led to sacrifice, whereas the volume of the anti-AM antibody-treated tumors was stabilized and reached a 70% decrease when compared with the controls (Figure 9). The mean tumor weights in the controls and the anti-AM antibody-treated groups were 4.05 and 1 g, respectively. A further suppression of tumor growth up to 98% was observed 70 days after treatment. The antitumor effect of the anti-AM antibody on U87 xenografts *in vivo* was most likely because of a direct action on the tumor through the suppression of AM:receptor interaction. Thus, the inhibition of the action of endogenous AM seems to be a key step in a complex cascade of events that result in tumor inhibition. It can, however, not be totally excluded that part of the *in vivo* response to the anti-AM antibody observed, is because of antibody-mediated complement

activation. The inhibitory effect of the antibody *in vitro* speaks against this possibility because the *in vitro* experiments were done with serum-free medium. SW1088 and U373 cells, which showed growth inhibition after treatment with anti-AM antibody *in vitro*, did not grow significantly in nude mice and, thus, the antitumor activity of anti-AM antibody on these cell lines *in vivo* could not be evaluated.

AM has been shown to be angiogenic in the chick chorioallantoic membrane assay and to increase human umbilical vein endothelial cell number.[16] At day 21 after treatment, more than 90% of the vessels on the tumor surface disappeared after anti-AM antibody treatment. The vessel area in the controls were significantly larger than those in the antibody-treated groups ($P < 0.0001$; Figure 10, A and B). Our data demonstrate that the density of vessels with lumen was decreased in the antibody-treated tumors despite the fact that there is no significant difference in cells stained positive for FVIII-related antigen (Figure 10, A and B) suggesting that AM might be involved in neovascularization and/or vessel stabilization. In accordance with these results, we could demonstrate increased apoptosis and decreased proliferation of anti-AM antibody-treated tumors (Figure 10C).

Taken together our results suggest that the anti-AM antibody treatment exhibits an anti tumoral effect through both tumoral and endothelial cells. To establish whether the autocrine/paracrine stimulation is a general feature of primary glioblastoma, the extension of these investigations to additional cell lines and the screening of primary tumors for the production of biologically active AM are required. Although our studies used polyclonal anti-peptide antibody to inhibit AM action, it is possible that growth-suppressive effects could be obtained using anti-receptor antibodies or efficient and potent AM antagonists.

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Regulation of Insulin Secretion and Blood Glucose Metabolism by Adrenomedullin

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ABSTRACT

Adrenomedullin (AM), a recently discovered hypotensive peptide, is expressed in the endocrine pancreas of different species, as demonstrated by immunocytochemistry. Electron microscopic studies with double immunogold showed colocalization of AM and pancreatic polypeptide. A homogeneous expression of AM receptor was found throughout the islet using *in situ* hybridization. Six different insulin-producing cell lines have been analyzed by reverse transcription-PCR and showed expression of both AM and its receptor. Two experimental models have been used to study the effects of AM in pancreatic physiology. 1) Analysis of isolated rat islets shows that AM inhibits insulin secretion in a dose-dependent manner. The monoclonal antibody MoAb-G6, which neutralizes AM bioactivity, was able to increase insulin release 5-fold; this effect was reversed by the addition of synthetic AM. 2) Oral glucose tolerance tests showed that iv injection of AM reduces the levels of insulin in the bloodstream with a concomitant increase in circulating glucose. These studies implicate AM as a newly defined factor of the insulin regulatory system that could be involved in disorders such as diabetes and obesity. (*Endocrinology* **137:** 2626–2632, 1996)

ADRENOMEDULLIN (AM), a recently characterized regulatory peptide (1–3), is generated from a larger 185-amino acid preprohormone through consecutive enzymatic cleavage and amidation. This process culminates in the liberation of a 52-amino acid bioactive peptide (4). AM has been found in numerous organs, including adrenal gland, heart atrium, kidney, and lung (2, 5). AM's role as a vasodilatory agent has been extensively studied (6–9). It acts through specific receptors (10) in the plasma membrane to activate adenylate cyclase activity and modulate Ca^{2+} flux in the target cells (11, 12). These signal transduction pathways are involved in numerous physiological processes, including the regulation of hormone secretion (13). It is well established that regulation of intracellular cAMP modulates hormone release in the pancreas (14, 15). As AM and its gene related peptide have been reported to influence the secretion rate of several hormones, including catecholamine (16), ACTH (17), and aldosterone (18), we investigated the potential role of AM in regulating the endocrine physiology of the pancreas.

Materials and Methods

Tissues

Human pancreas sections were obtained from Dako Corp. (Carpinteria, CA). Pancreata from rats, hamsters, guinea pigs, cats, and dogs were fixed in Bouin's fluid (Sigma Chemical Co., St. Louis, MO) and embedded in paraffin. Animal tissues were obtained through a contract with Science Application International Corp., located at the Frederick Cancer Research and Development Center (Frederick, MD).

Antibodies

A previously characterized antibody against P072, a fragment of AM (5), was used for immunocytochemistry together with commercially available antisera against insulin, glucagon, somatostatin, and pancreatic polypeptide (Accurate Chemical and Scientific Corp., Westbury, NY).

A monoclonal antibody, designated MoAb-G6, was developed against the P072 peptide of AM following a procedure modified from a previously described methodology (19). In brief, BALB/c mice were hyperimmunized with P072 peptide conjugated to keyhole limpet hemocyanin via glutaraldehyde cross-linkage (1 mg/1 mg coupling ratio). Splenic lymphocytes were fused to mouse myeloma cell line RNS1 following standard protocols. Resulting hybrids were screened for anti-P072 MoAb production using a solid phase enzyme-linked immunosorbent assay technique. Responding hybridomas were subcloned twice, expanded in mass culture, and used as the seed stock for ascites generation. MoAb-G6 (IgAκ isotype) represented the highest titered antibody and was purified from ascites fluid by affinity chromatography on solid phase P072-coupled resin (Affi-Prep 10, Bio-Rad Laboratories, Richmond, CA; coupling efficiency, 10 μmol peptide/ml resin). The antibody was characterized for binding specificity using a solid phase RIA with [^{125}I]protein A as the detector (20) (Fig. 1). In brief, test peptides were passively absorbed to individual wells (50 ng/well; overnight at 4 C) of a 96-well polyvinylchloride microtiter plate (Dynatech Laboratories, Chantilly, VA), after which the plate was coated with 1% BSA in PBS to minimize nonspecific binding. Test peptides (Peninsula Laboratories, Belmont, CA) included AM, P072, calcitonin gene-related peptide (CGRP), gastrin-releasing peptide, glucagon-like peptide 1, vasoactive intestinal peptide, arginine vasopressin, GRF, cholecystokinin, amylin, gastrin, oxytocin, calcitonin, αMSH, pancreatic polypeptide, peptide tyrosine-tyrosine, *Tabanus atratus* hypotrehalosemic hormone,

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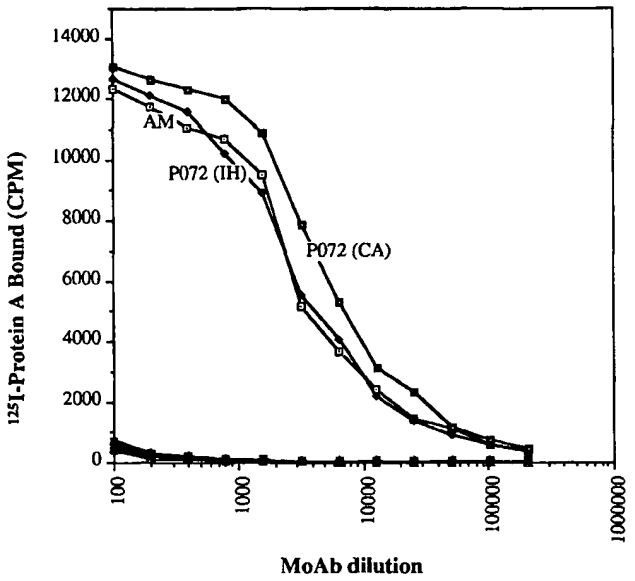

FIG. 1. Titration curve for monoclonal antibody MoAb-G6 binding to solid phase test peptides. The antibody recognized AM and the immunogen P072; both our in-house peptide (IH) and a commercially available product (CA, Peninsula Laboratories). All other target peptides [P070, CGRP, gastrin-releasing peptide (GRP), glucagon-like peptide 1 (GLP 1), vasoactive intestinal peptide (VIP), arginine vasopressin (AVP), GRF, cholecystokinin (CCK), amylin, gastrin, oxytocin, calcitonin, αMSH, pancreatic polypeptide, peptide tyrosine-tyrosine (PYY), *Tabanus atratus* hypotrehalosemic hormone, and BSA] showed negligible binding.

and BSA (Sigma Chemical Co., St. Louis, MO) as a negative control. The titration screen covered a range from 1:100 to 1:204,800 (2-fold dilutions).

Immunocytochemistry (light and electron microscopies)

The avidin-biotin-peroxidase complex method (21) was used for paraffin sections. Negative controls included substitution of the primary antibody with preimmune serum from the same rabbit and preabsorption of the antibody with 10 nmol/ml of the synthetic peptide (AM or CGRP).

For immunoelectron microscopy, three rats were perfused with a mixture of 2.5% paraformaldehyde and 2.0% glutaraldehyde in cacodylate buffer. Small pieces of pancreas were dehydrated and embedded in resin. Ultrathin sections were mounted in nickel grids, and the double immunogold method was used as previously described (22). In brief, sections were incubated in 1% BSA in TBS for 30 min, followed by an overnight incubation at 4 C with a mixture of the antibodies at the optimal dilution. Subsequent steps, all at room temperature, included rinses in 1% BSA-TBS, incubation with the gold-labeled secondary antibodies, rinses with BSA-TBS and distilled water, and double staining in 5% aqueous uranyl acetate and lead hydroxide. In addition to the controls used in light microscopy, one of the primary antisera was omitted in serial sections to exclude possible interactions.

In situ hybridization

Detection of the AM receptor (AM-R) messenger RNA (mRNA) was performed using *in situ* hybridization, as previously described (23). The full-length complementary DNA was ligated into the expression vector pcDNA1 (10) and used to generate riboprobes. The plasmid was linearized with *Eco*RV and *Bam*HI and was used as a template to synthesize digoxigenin-labeled sense and antisense RNA transcripts. Hybridization was performed in a moist chamber at 46 C for 20 h in a 20-μl volume containing 2.5 ng probe/μl. After stringency washes, visualization of digoxigenin was performed using the Digoxigenin detection kit (Boehringer Mannheim, Indianapolis, IN). Controls included the use of the sense probe and digestion with ribonuclease before hybridization.

Cell culture

Six well characterized, insulin-producing cell lines (RINm, N289, TR4, CRL 2057, CRL 1777, and CRL 2055) were obtained from the American Type Culture Collection (Rockville, MD). A small cell lung carcinoma, H187, was obtained from the NCI-Navy Medical Oncology Branch and was used as a negative control for AM-R expression.

Reverse transcription-PCR (RT-PCR) and Southern blot

Polyadenylated mRNA from human normal tissue (adrenals, pancreas, and thymus) was purchased from Clontech (Palo Alto, CA). The Micro-Fast Track kit (Invitrogen, San Diego, CA) was used to extract mRNA from cell lines. RT-PCR and Southern blot were carried out as previously described (5). A set of primers that recognizes the most conserved regions of the AM gene were designed and are shown in Table 1, which also contains the sequences for the oligonucleotides used to amplify the AM-R mRNA. Base sequence analysis of isolated bands confirmed identity of the RT-PCR products.

Islet isolation, insulin secretion, and cAMP assays

Islets from six Sprague-Dawley rats were isolated following well established protocols (24). Assays were performed in 24-well plates (90 islets/well). After a 45-min incubation in RPMI-1690 medium containing 5.6 mM glucose, a second incubation was performed in RPMI containing 20.6 mM glucose and various concentration of AM in the presence or absence of MoAb-G6 (2.5 μg/ml). Supernatants from both incubations were tested by RIA for insulin (Amersham, Arlington Heights, IL). After collecting the medium, the islets from the same experiments were saved for analysis of cAMP contents (25). Islets were extracted in 50% ethanol and centrifuged, and the supernatants were tested for cAMP using a RIA kit (New England Nuclear Corp., Boston, MA) following manufacturer's instructions. The experiments were repeated three times.

Insulin secretion and cAMP assays in cultured β-cells

Insulin-producing cell lines were seeded in 24-well plates at 2×10^5 cells/well, and analyses of insulin secretion and cAMP were performed as described above.

In vivo studies

Glucose tolerance methods have been previously described (26). Six Sprague-Dawley rats were administered a glucose solution (400

TABLE 1. Sequences of the oligonucleotides synthesized for this study

Adrenomedullin	
Sense (AM 250–270)	5′-AAG-AAG-TGG-AAT-AAG-TGG-GCT-3′
Antisense (AM 523–542)	5′-TGT-GAA-CTG-GTA-GAT-CTG-GT-3′
Probe (AM 430–450)	5′-TCT-GGC-GGT-AGC-GCT-TGA-CTC-3′
Adrenomedullin receptor	
Sense (AM-R 476–497)	5′-AGC-GCC-ACC-AGC-ACC-GAA-TAC-G-3′
Antisense (AM-R 923–946)	5′-AGA-GGA-TGG-GGT-TGG-CGA-CAC-AGT-3′
Probe (AM-R 788–811)	5′-GGT-AGG-GCA-GCC-AGC-AGA-TGA-CAA-3′

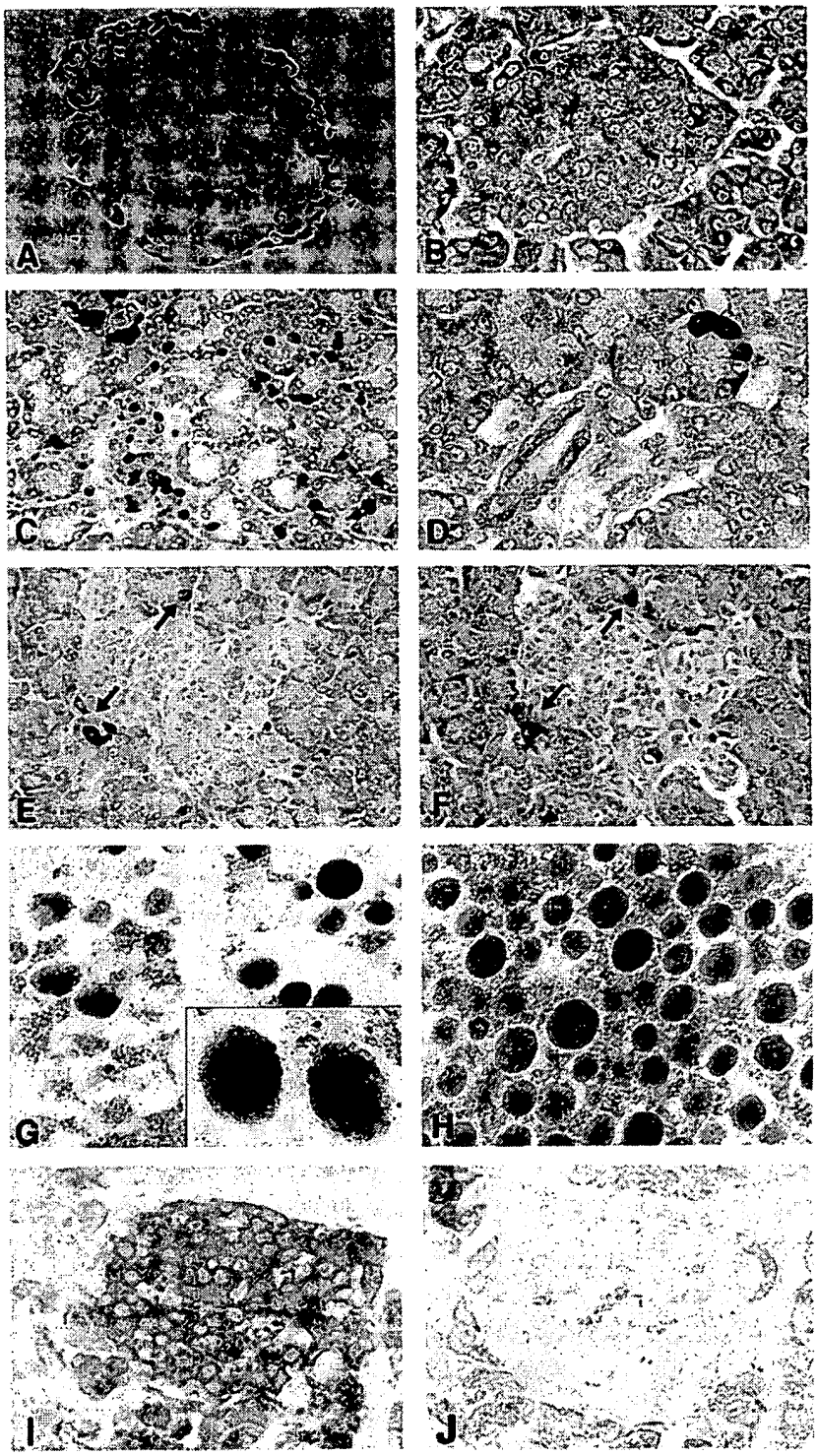

FIG. 2. Distribution of AM in the pancreas, as shown by immunocytochemistry. A, Rat pancreas shows mild immunoreactivity throughout the entire islet of Langerhans and strongly stained cells in the periphery. No counterstaining was applied to this section. B, Hamster pancreas displays a similar pattern. Magnification, ×370. C, Dog pancreas contains numerous immunoreactive cells scattered throughout the parenchyma. Magnification, ×180. D, Immunoreactivity in ductal system of guinea pig pancreas. Note that endocrine cells are more intensely stained than duct cells. Magnification, ×370. Serial sections of hamster pancreas immunostained for AM (E) and pancreatic polypeptide (F) show colocalization of both immunoreactivities (*arrows*). Magnification, ×180. G, Double immunogold staining by electron microscopy in rat pancreas shows colocalization of AM (small gold particles, 10 nm) and pancreatic polypeptide (large gold particles, 20 nm) in the cell situated to the left. A small fragment of a negative α-cell can be observed to the right. Magnification, ×27,000. The *inset* shows two secretory granules containing both immunoreactivities at higher magnification. Magnification, ×62,000. H, Detail of a D cell showing some immunoreactivity for AM (large particles) in the somatostatin-containing (small particles) secretory granules. Magnification, ×27,000. I, *In situ* hybridization for AM-R in rat pancreas with the antisense probe; J, negative control using the sense probe. Magnification, ×180.

mg/100 g BW) via gastric intubation (protocol 95–062, NCI). Ocular blood samples were collected at intervals after glucose loading, and the glucose concentration was determined by a colorimetric assay (Sigma). Blood insulin levels were determined on the same samples by RIA (Amersham). Three days later, the same rats received 1 μl AM (60 μM)/g BW through iv injection immediately after glucose administration, and glucose tolerance tests were repeated. The overall effects of AM on plasma glucose and insulin responses to oral glucose gavage were initially compared by ANOVA (Proc Anova, 1986, SAS Institute, Cary, NC). To satisfy the basic statistical assumptions for the proper use of ANOVA, animals with missing data cells were dropped from the computations. Because of significant treatment by time interactions in the ANOVA, data were further analyzed by regression analysis using a general linear model (Proc GLM, 1986, SAS) to compare differences in treatment means at specific time points using all animals. Furthermore, in this analysis, differences by

treatment in the time to peak response after glucose gavage and the areas under the insulin and glucose response curves were compared.

Results

Distribution of AM in the pancreas

In humans, rats, and hamsters, the islets of Langerhans were immunoreactive for AM in all cells. However, specific cells in the periphery presented a stronger positivity than others (Fig. 2, A and B). In addition, a few strongly stained cells were found scattered through the pancreatic parenchyma or among the ductal epithelial cells. In the guinea pig, cat, and dog pancreata, the staining pattern was different. Most of the AM-like cells were scattered in the parenchyma, and only occasional immunoreactive cells were found in the periphery of the islets. In these cases, no immunoreactivity was evident in the β-cells (Fig. 2C). In addition, low intensity staining was consistently found in the ductal epithelia of the guinea pig pancreas (Fig. 2D).

To further characterize the nature of the cells containing the AM-like material, serial sections were stained with antibodies against AM and the major pancreatic hormones. Where weak immunoreactivity for AM was noted throughout the islet, colocalization with all other hormones was evident, but the cells strongly positive for AM colocalized only with pancreatic polypeptide (Fig. 2, E and F). Double immunogold staining at the electron microscopic level confirmed the colocalization of AM with pancreatic polypeptide in peripheral cells of rat pancreas (Fig. 2G). Consistent with the light immunohistochemical data, few immunogold particles detecting AM were found in other endocrine cell types by electron microscopy (Fig. 2H).

Distribution of the AM-R was determined by *in situ* hybridization in paraffin sections of rat pancreas. A homogeneous distribution of the mRNA was observed throughout the islets of Langerhans (Fig. 2, I and J).

We were able to confirm these morphological results with molecular techniques. Six insulin-producing cell lines showed expression of mRNA for both the ligand and the receptor (Fig. 3).

Physiological effects of AM in endocrine pancreas regulation

The addition of AM to freshly isolated islets resulted in a dose-dependent reduction of insulin secretion (Fig. 4A). This inhibition reached 78% for an AM concentration of 1 μM. Using the neutralizing monoclonal antibody MoAb-G6, we observed a dramatic 5-fold increase in insulin secretion in the absence of extrinsic AM (Fig. 4A). The addition of extrinsic AM again resulted in a dose-dependent competitive inhibition (Fig. 4A). Consistent with this observation, cAMP levels increased in the islets when AM was added (Fig. 4B). Similar studies were performed in β-cell lines in culture, and no variation was observed in either insulin secretion or cAMP content after the addition of AM (results not shown).

In another experiment we measured the influence of AM on oral glucose tolerance testing of nonanesthetized rats (Fig. 5). In control animals, the plasma insulin concentration increased rapidly to a peak 20 min after glucose administration and remained elevated until 60 min (Fig. 5A). In contrast, rats

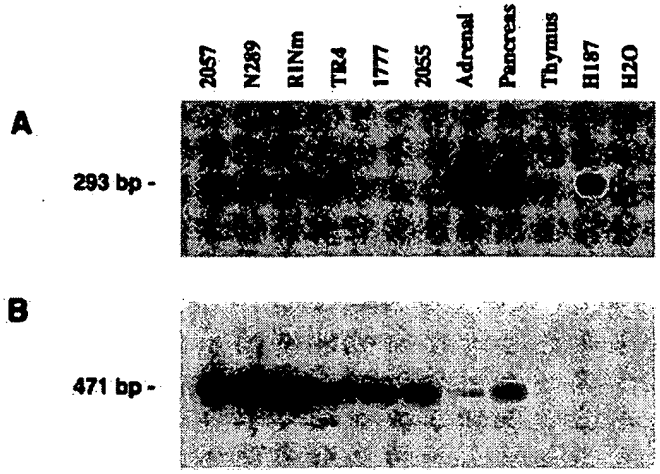

FIG. 3. Southern blot for AM (A) and AM-R (B) in six cell lines expressing insulin and in human adrenal and pancreas mRNA. Human thymus and the cell line H187 (small cell lung carcinoma) were included as negative controls for AM and AM-R, respectively.

treated with AM did not begin to respond to glucose until 40 min, with a peak response 60 min after glucose treatment. In addition, plasma insulin concentrations decreased 20 min ($P < 0.06$) after glucose challenge and tended to be greater than control values at 60 min ($P < 0.06$). Twenty minutes after glucose administration, plasma concentrations of insulin were approximately 2-fold greater in control rats *vs.* AM-treated rats ($P < 0.009$). Plasma glucose concentrations in control rats increases uniformly over time, peaking 4–6 h after feeding (Fig. 5B). In association with the depressed insulin response, plasma glucose levels in AM-treated rats increased rapidly to peak 1 h postoral glucose and decreased in association with a progressive change in plasma glucose. The time to the glucose peak was significantly different between control and AM-treated rats ($P < 0.0025$). The difference between treatments at 1 h was also highly significant ($P < 0.005$).

Discussion

Our morphological data show colocalization of AM with pancreatic polypeptide in the islets of Langerhans. This result differs from the findings of a previous report (27), which suggested a colocalization with somatostatin based only on the peripheral distribution of AM immunoreactivity.

AM has some structural similarities to CGRP and amylin (28), both of which are involved in pancreatic physiology. Nevertheless, the distribution of these three peptides in the pancreas varies. Amylin is mainly located in the β-cells, colocalizing with insulin (29), and CGRP is present in pancreatic nerves as well as in D cells of the islets (30, 31). The differential distribution of the immunoreactivities together with the absorption controls excludes a possible cross-reactivity of the AM antibody with amylin or CGRP.

We have shown, by *in situ* hybridization analysis, that the AM-R mRNA is homogeneously distributed throughout the islet. These data suggest that all endocrine cell types of the pancreas have an inherent potential for expressing re-

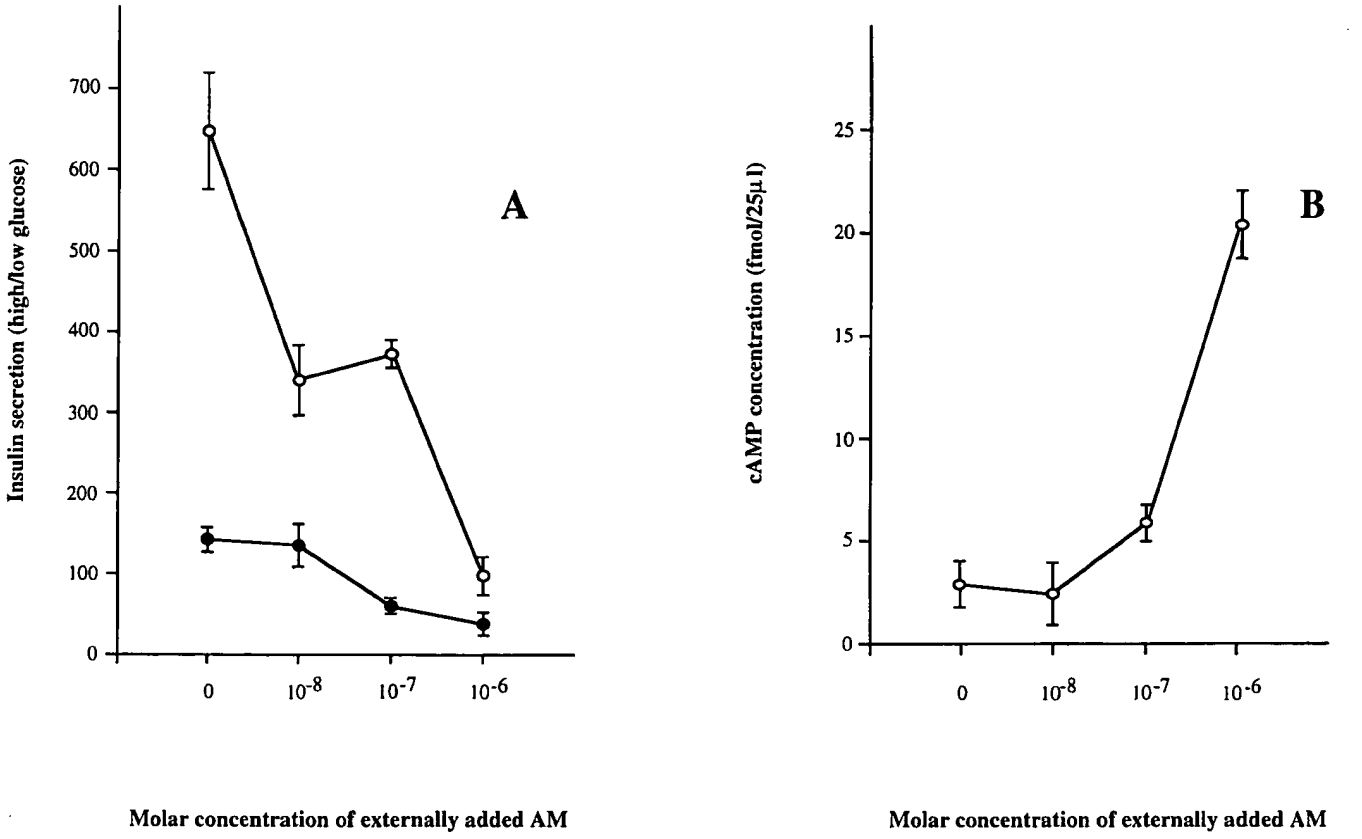

FIG. 4. Effects of AM and MoAb-G6 (α-AM) on the release of insulin from rat isolated islets. A, Increasing concentrations of AM reduce insulin secretion in the presence (○) or absence (●) of MoAb-G6 antibody. Note the dramatic increase in insulin secretion mediated by the antibody. The ratio between the insulin concentration found in the high glucose supernatant and that measured in the low glucose solution is represented on the *ordinates*. As a reference, absolute insulin concentrations in the wells without AM or MoAb-G6 were 0.08 ± 0.01 ng/ml (low glucose) and 10.5 ± 0.6 ng/ml (high glucose). With the addition of the antibody, these values were 0.10 ± 0.02 ng/ml (low glucose) and 62.5 ± 3.2 ng/ml (high glucose). B, Dose-dependent increase in intracellular cAMP in the islets after the addition of AM. Data are the mean ± SD of two wells.

ceptor protein, and hence, certain aspects of their functional role may be regulated by AM. It should be noted, however, that although β-cell lines expressed AM-R, as analyzed by RT-PCR, none responded to exogenous AM treatment by insulin release or cAMP production. Thus, translational regulation of receptor protein expression may play a critical role in mediating the biological effects of AM. On the other hand, we had previously demonstrated expression of AM in lung tumors (5), and the presence of this peptide-receptor system in insulinomas could be more related to the neoplastic phenotype than to the original source of the tumor.

Our experimental data on isolated islets clearly demonstrate the inhibitory role of AM on insulin secretion. AM has been previously described as an antisecretagogue in different systems (16–18), and our study with isolated islets is but another example of this ability. These results are further strengthened by the results observed with the neutralizing monoclonal antibody MoAb-G6, which blocked both endogenous and exogenous AM effects on insulin secretion. Such studies implicate the existence of a continuous inhibitory tone in the islet that may contribute to pancreatic homeostasis.

It has been previously shown that AM triggers increases in both cAMP and Ca^{2+} flux in the target cell (11, 12), and we have been able to demonstrate an increase in cAMP in the islets. However, no response to AM was observed in cultured β-cells even though expression of AM-R was detected by RT-PCR. These results, in agreement with previous studies showing that substances able to increase cAMP and Ca^{2+} in β-cells induce insulin secretion rather than inhibit it (32), indicate that the regulation of insulin secretion through AM is not directly processed in the β-cell; rather, more complex interactions, involving other endocrine cell types, take place at the islet level. A similar mechanism has been described for amylin (33), and further investigation is needed to determine which cell type is responsible for these actions. The apparent contradiction in β-cells between expression of AM-R and lack of cAMP response could be explained by the presence of phosphodiesterases; these enzymes regulate levels of intracellular cAMP by catalyzing its degradation and have been found in the islets of Langerhans (34). Other possibilities consist of AM acting through a different intracellular pathway or through other receptors. There is evidence that AM binds to CGRP receptors with lower affinity (11, 35, 36) and could bind amylin receptors similarly, because the three peptides are structurally related (28).

The *in vivo* experiments fully supported the observations made in isolated islets; AM was able to attenuate and delay

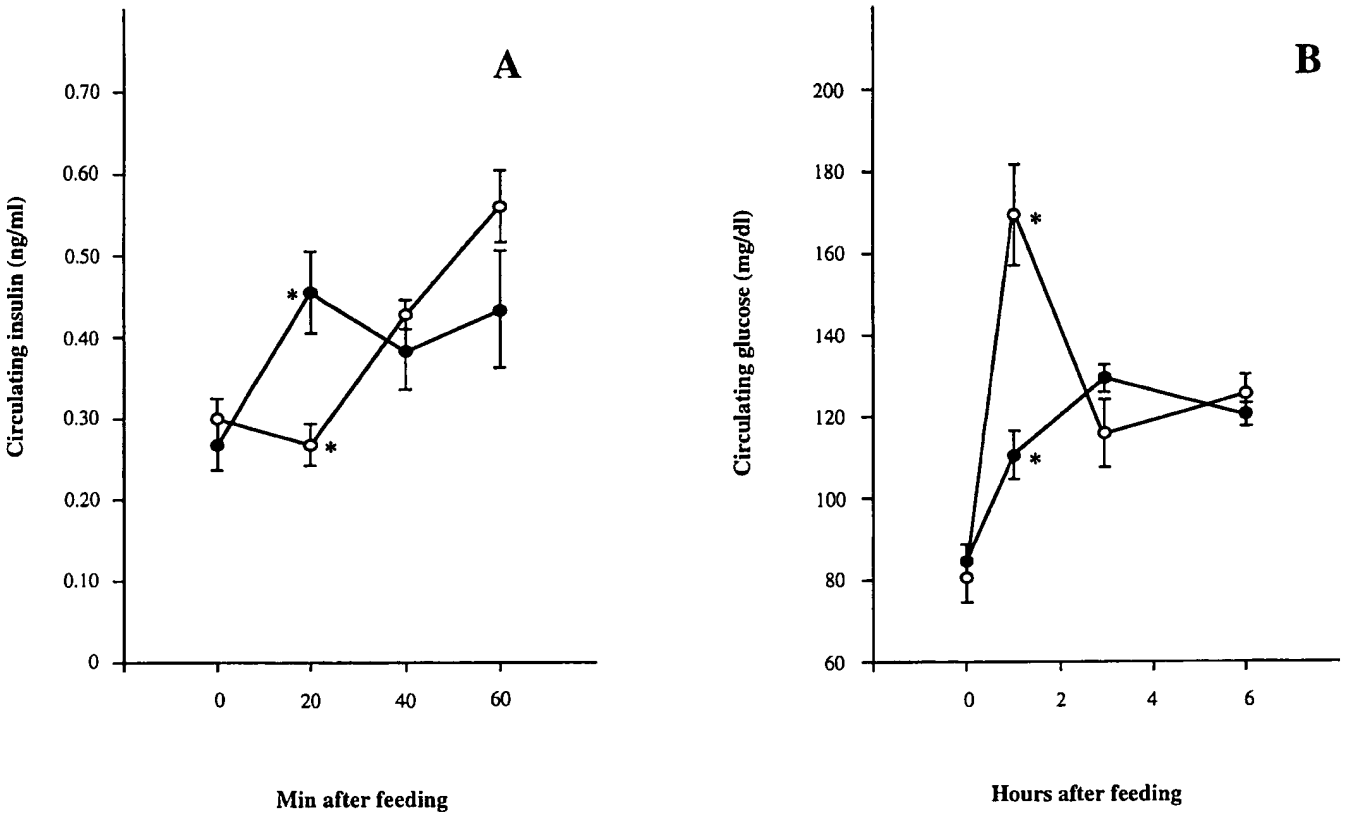

FIG. 5. Glucose tolerance tests were performed on Sprague-Dawley rats (250–300 g) in the presence (○) or absence (●) of AM. A, Significant differences ($P < 0.01$) in insulin levels were observed 20 min after iv injection of AM. B, The difference in levels of circulating glucose was also highly significant ($P < 0.005$) 1 h after injection. Data are the mean ± SD for six animals. *, Statistical significance ($P < 0.01$).

the insulin response to oral glucose challenge, resulting in elevated glucose levels early in the response. The well known vasodilatory effect of AM may also influence the insulin secretion rate by increasing pancreatic perfusion. Although this cannot be the main mechanism, as demonstrated in the islet experiments where blood flux is irrelevant, it merits further evaluation.

As the expression of AM (at least in the cardiovascular system) is affected by levels of tumor necrosis factor-α, interleukin-1, lipopolysaccharide, interferon-γ, endothelin-1, angiotensin II, substance P, bradykinin, thrombin, and vasoactive intestinal peptide (37), any combination of these bioactive substances may be involved with AM in regulating insulin secretion. In fact, some reports link different forms of diabetes with these substances (38, 39).

It has also been observed that AM's physiological effects are somehow connected with those produced by nitric oxide (9). This relationship may be due to the cross-talk in the target cell between the signal transduction pathways for AM, which increases cAMP, and those for nitric oxide, which increases cGMP (40). As nitric oxide synthase is present in the islets of Langerhans, and nitric oxide regulates insulin secretion (41, 42), it would be interesting to investigate the interactions between these two regulatory systems.

A better understanding of the interactions of AM in normal pancreatic physiology and in different pathological states, such as diabetes and obesity, may help define new areas of therapeutic intervention to obliterate these metabolic disorders.

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P-glycoprotein suggests that this region is relatively more immunogenic than most of the protein. The C-terminus possibly is part of a hydrophilic domain in an otherwise relatively hydrophobic protein. Alternatively, it may be a region of the polypeptide that is conformationally more fluid and, therefore, is more likely to be antigenic[18-20]. It is significant that all eight monoclonal antibodies showed specificity for the same plasma membrane component, supporting the notion that the expression of P-glycoprotein is the major alteration of the multidrug-resistant cell surface. One of the epitopes described here (identified by group I antibodies) seems to be highly conserved among species, but the other two appear to be expressed in a species-dependent manner, as seen in Fig. 1. The latter epitopes may represent more variable regions of P-glycoprotein structure.

The monoclonal antibodies described here will serve as sensitive and specific reagents for the detection of P-glycoprotein in experimental systems and in normal and neoplastic tissues. Furthermore, they have potential for use in screening human tumour biopsies, where early detection of elevated levels of P-glycoprotein might indicate the need for a departure from routine chemotherapy and the application of novel treatments to circumvent the multidrug resistance phenotype[7,21,22]. Preliminary findings of elevated amounts of P-glycoprotein in some ovarian carcinomas of patients failing to respond to chemotherapy have already demonstrated the usefulness of the present monoclonal antibodies for this purpose[16].

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Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer

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The autocrine hypothesis proposes that a cell produces and secretes a hormone-like substance that can interact with specific membrane receptors on its surface to induce effects such as proliferation[1]. Thus, a cancer cell could act to stimulate its own growth. Bombesin and bombesin-like peptides (BLPs) such as gastrin-releasing peptide (GRP) cause various physiological responses in mammals[2], including stimulation of proliferation of 3T3 mouse fibroblasts[3] and normal human bronchial epithelial cells *in vitro*[4] and induction of gastrin cell hyperplasia and increased pancreatic DNA content *in vivo* in rats[5,6]. Human small-cell lung cancer (SCLC) cell lines produce and secrete BLPs[7-12] and can express a single class of high-affinity receptors for BLPs[13]. Exogenously added BLPs can also stimulate the clonal growth and DNA synthesis of SCLC *in vitro*[14,15]. These findings suggest that BLPs function as autocrine growth factors for this tumour. One way to test this hypothesis is to interrupt the function of the endogenously produced BLPs. Here, we demonstrate that a monoclonal antibody to bombesin binds to the C-terminal region of BLPs, blocks the binding of the hormone to cellular receptors and inhibits the clonal growth of SCLC *in vitro* and the growth of SCLC xenografts *in vivo*. These results demonstrate that BLPs can function as autocrine growth factors for human SCLC.

The C-terminal region of bombesin and GRP, which competes effectively for the same class of high-affinity receptors on pituitary cells and SCLC membranes, is required for receptor binding and physiological activity[13,16,17]. Murine monoclonal antibodies were prepared against a synthetic analogue of amphibian bombesin (Lys 3-bombesin) which has the same C-terminal heptapeptide sequence as human and porcine GRP[18,19]. The neuropeptide substance P has the same carboxy-terminal Leu-Met dipeptide as bombesin but is rarely found in SCLC and binds to a different set of receptors than bombesin[7,10]. Thus, we screened for monoclonal antibodies that bound to bombesin but not substance P. One anti-bombesin monoclonal antibody, 2A11 (IgG_1, κ), showed no cross-reactivity with substance P and was purified from ascites for study.

Using solid-phase synthetic peptides as targets in a radioimmunoassay (RIA), the 2A11 antibody bound to bombesin, the Lys 3 derivative of bombesin used for immunization, the Tyr 4 derivitive of bombesin used for iodination, GRP 1-27, GRP 14-27 and GRP 20-27, whereas GRP 22-27, GRP 1-16, substance P and other tachykinins[20] showed insignificant 2A11 binding despite some C-terminal homology with bombesin and GRP (Table 1). In addition, a series of unrelated peptides that can be produced by SCLC, such as calcitonin, adrenocorticotropic hormone (ACTH), arginine vasopressin (AVP) and neurotensin did not bind 2A11. Note that, although SCLCs make BLPs, the 2A11 antibody did not bind to live, intact SCLC (Table 1).

We next used 2A11 to develop a quantitative, competitive RIA system for BLPs using solid-phase 2A11 and ^{125}I-labelled Tyr 4-bombesin. This assay was able to detect BLPs in the range of 5-100 pg per well and BLPs were detected by the RIA in extracts of all 10 SCLC lines tested (Table 2). These values are in good agreement with previously reported quantitative data on many of the same tumour lines using rabbit anti-bombesin heteroantisera[7]. In contrast, none of the non-SCLC lines

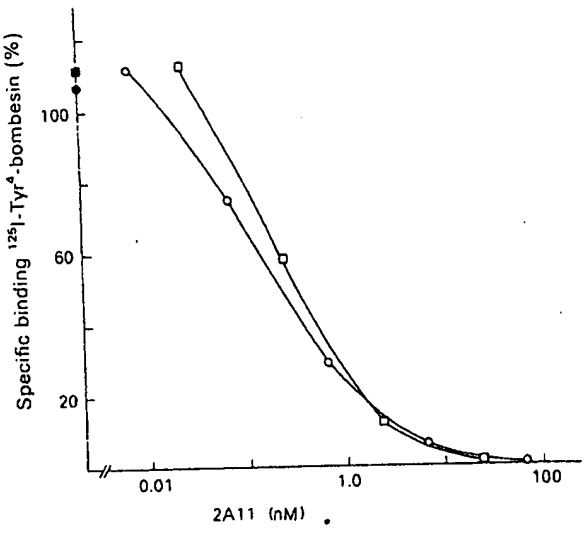

Fig. 1 Inhibition of binding of labelled bombesin in a radioreceptor assay by monoclonal antibody 2A11 using rat brain membranes (□)[16] or small-cell lung cancer (NCI-H345) cell (○) preparations[13]. The black symbols on the ordinate show that 67 nM MOPC 21 control immunoglobulin did not inhibit the binding of the labelled peptide to either receptor preparation.
Methods. ^{125}I-Tyr 4-bombesin (50–80 μCi μg^{-1}, 40,000 c.p.m. per test) was incubated with the indicated concentrations of purified monoclonal antibody 2A11 for 60 min at 25°. Receptor binding assays were conducted using 10 mg wet weight of crude rat brain homogenate or 2×10^6 cell of SCLC line NCI-H345 as described previously[16,30]. The difference in binding in the absence and presence of an excess unlabelled bombesin (1 μM) was taken as a measure of specific binding.

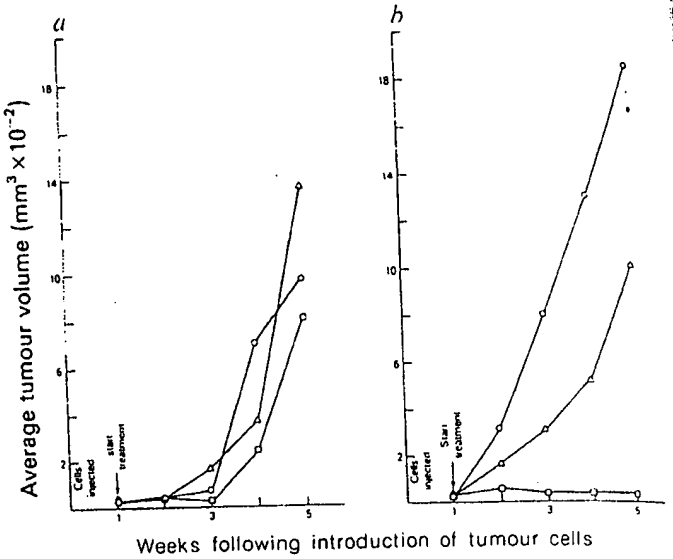

Fig. 2 Effect of treatment with monoclonal antibody 2A11 (□), control monoclonal antibody MOPC 21 (△) or PBS (○) on growth of human tumour xenografts in athymic nude mice. *a*, Human malignant melanoma cell line NCI-H234A; *b*, human small-cell lung cancer line NCI-N592.
Methods. Tumour cells (10^7/mouse) were subcutaneously injected into the flanks of athymic nude mice (BALB/c *nu/nu*) and a palpable mass formed after 7 days. Treatment begun on day 7 consisted of three trial groups (5 mice per group), each given 0.5 ml i.p. injections three times a week of either PBS (○), 200 μg purified MOPC 21 immunoglobulin (△) or 200 μg of purified 2A11 antibody (□). Thus, each mouse in the immunoglobulin-treated groups received a total of 600 μg immunoglobulin per week for four weeks. Tumours were measured once a week. Tumour growth is reported as an average relative tumour volume, s.e.m. ±10%.

(adenocarcinoma, large-cell and squamous cell lung carcinoma) or the non-lung cell lines (B-lymphoblastoid, T-cell lymphoma and melanoma) expressed detectable levels of BLPs (<0.01 pmol mg^{-1} acid soluble protein) (Table 2).

As 2A11 bound to the C-terminal region of BLPs (such as GRP 14–27 and GRP 20–27), we tested the ability of the antibody to block the binding of labelled bombesin to its cell-surface receptor on small-cell lung cancer and rat brain membranes (Fig. 1). Antibody 2A11 blocked bombesin–receptor interaction in a dose-related fashion with both receptor preparations (Fig. 1). Almost complete inhibition of bombesin binding (using 12–18 nM labelled bombesin) occurred with 10 nM 2A11. An indifferent mouse myeloma protein, MOPC 21, of the same isotype as 2A11 (IgG$_1$ κ) at 67 nM did not significantly affect bombesin–receptor interaction (<1% inhibition).

Antibody 2A11 was then examined for its effect on the *in vitro* growth of SCLC cell lines using an agarose cloning assay. Two types of SCLC cell lines were tested against various concentrations of 2A11. The first was a 'classic' SCLC line (NCI-N592) with typical SCLC morphology (growth in tight balls in suspension, scant cytoplasm) and high specific activities of L-dopa decarboxylase[21]. The second was a 'variant' SCLC line (NCI-N417) that deviates morphologically (growth as loose chains, with large cell cytology and abundant cytoplasm) from typical SCLC, has undetectable levels of L-dopa decarboxylase, grows rapidly, clones with high efficiency and is amplified for the c-*myc* oncogene[21,22]. Both cell lines produce detectable levels of bombesin (Table 2) and both have the SCLC features of high specific activities of the BB isozyme of creatine kinase and neurone-specific enolase[21,23]. The clonogenic growth of both cell lines was markedly inhibited by ≥3 μg ml^{-1} 2A11 (Table 3). In contrast, 10 μg ml^{-1} of the isotype-matched mouse myeloma protein MOPC 21 or boiled 2A11 showed no significant inhibition of SCLC growth in the same assay (Table 3). Exogenously added synthetic bombesin can stimulate the clonal growth of SCLC in serum-free medium (Table 3)[14]. The addition of 50 nM of exogenous synthetic bombesin added simultaneously with 2A11 completely prevented the 2A11 growth inhibition demonstrating the specificity of the 2A11 effect (Table 3).

Fig. 3 Representative nude mice 5 weeks after xenografting with small-cell lung cancer NCI-N592 and treated with MOPC 21 (mouse 1), PBS (mouse 2) or 2A11 (mouse 3). Arrows point to the site of tumour innoculation. Large tumours are seen for the two controls (1 and 2) and no tumour mass was detected in mouse 3 at week 5.

Nude mouse heterotransplant studies were used to investigate the ability of 2A11 to inhibit SCLC growth *in vivo*. Two tumour cell lines were used in this study, the classic SCLC line NCI-N592 and a melanoma cell line, NCI-H234A, which did not make BLP and which was unresponsive to bombesin *in vitro*[14], to serve as a control. One week after their inoculation into nude

Table 1 Immunological cross-reactivity of solid-phase synthetic peptides with bombesin using the 2A11 binding assay

Peptide target	Cross-reactivity (%)
Bombesin-like peptides	
Bombesin (BN)	100
Lys 3-BN	98
Tyr 4-BN	96
Gastrin-releasing peptide (GRP 1-27)	102
GRP 14-27	102
GRP 20-27	112
GRP 1-16	0.26
GRP 22-27	<0.02
Tachykinins	
Substance P, physalaemin, kassinin, eledoisin	all <0.02
Unrelated peptides	
Calcitonin, vasoactive intestinal peptide, motilin, β-endorphin, leu-enkephalin, glucagon, somatostatin, neurotensin, xenopsin, ACTH, AVP	all <0.07
Bovine serum albumin (BSA)	<0.02
Live small-cell lung cancer cells	<0.02

BALB/c mice were immunized subcutaneously three times with Lys 3-bombesin conjugated to BSA[24] (100 μg bombesin equivalents per injection; the first dose was in complete Freund's adjuvant, the last two in incomplete Freund's). Monoclonal antibodies were derived by standard methods following fusion to the mouse myeloma cell line X63-Ag8.653 (refs 25, 26). Hybrids were screened for anti-bombesin activity 10 days after fusion by indirect RIA using radio-autography on solid-phase target plates coated with bombesin, substance P or 1% BSA. Hybridomas producing antibodies binding to bombesin but not BSA or substance P were cloned several times. Monoclonal antibody 2A11 (IgG_1 κ) was grown in ascites, purified[27] and used for all subsequent tests. For solid-phase RIA, peptides (all purchased from Peninsula Laboratories) were adsorbed to 96-well polyvinyl chloride microtitre plates (Dynatech) by adding 0.05 ml 10 μg ml^{-1} peptide in PBS for 1 h, washing in PBS, then blocking with a 1% BSA/PBS solution. Porcine sequence synthetic GRP peptides were used[18]. Viable SCLC cell lines NCI-H128, NCI-H209 and NCI-H345 at 10^5 cells per well were tested in binding assays using 96-well plates with glass filters (V & P Scientific, San Diego)[28]. Purified 2A11 (10 μg ml^{-1}) was added, incubated and washed three times. Protein A affinity purified rabbit anti-mouse IgG (Cappel Laboratories) (1.6 μg ml^{-1}) was added, incubated, washed three times, then ^{125}I-protein A (Pharmacia, labelled by chloramine T method to 40-50 μCi $μg^{-1}$) 40,000 c.p.m. per well was added, incubated, washed eight times and the wells cut out and counted in a Beckman γ-counter. All incubations were for 1 h at room temperature in PBS with 1% BSA in 0.025 ml volumes. Percentage cross-reactivity = (c.p.m. ^{125}I-protein A bound on peptide target − c.p.m. bound on BSA target)/(c.p.m. bound by bombesin − BSA background ×100). The bombesin target caused 2A11 binding sufficient to bind 33,000 c.p.m. of ^{125}I-protein A whereas the BSA background bound 30 c.p.m. Mouse myeloma protein MOPC 21 (IgG_1 κ, Litton Bionetics) was used as a control at 10 μg ml^{-1} and did not bind to the peptides. All values are the average of triplicate determination and variation between replicates was <5%.

Table 2 Quantitation of BLPs in human cell lines using a competitive RIA with monoclonal antibody 2A11

Cell line providing extract	Immunoreactive BLP (pmol per mg acid-soluble protein)
Small-cell lung cancer lines	
NCI-H128	18.4
NCI-H209	17.4
NCI-H345	7.8
NCI-H510	5.4
NCI-N592	3.9
NCI-H187	3.7
NCI-H69	2.3
NCI-H146	1.6
NCI-N417	0.95
NCI-H446	0.13
NCI-H82	0.04
Non-small-cell lung cancer lines	
A549 (adenocarcinoma)	<0.01
NCI-H23 (adenocarcinoma)	<0.01
NCI-H125 (adenocarcinoma)	<0.01
NCI-H157 (large cell lung cancer)	<0.01
9812 (large cell lung cancer)	<0.01
U1752 (squamous cell lung cancer)	<0.01
Other human cell lines	
NCI-H128BL (B-lymphoblastoid)	<0.01
HCI-H78 (T-cell lymphoma)	<0.01
NCI-H234A (malignant melanoma)	<0.01

Cell lines were grown as described previously[21,23] and for preparation of cell extracts for RIA, $1-5\times10^7$ cells were washed three times in PBS and resuspended in 1 ml of 2 M acetic acid, homogenized, heated in a boiling water bath for 15 min, clarified by centrifugation at 3,000 r.p.m. and the supernatant lyophilized and stored at −80°. The freeze-dried extract was resuspended in 0.5-1 ml PBS, reclarified and protein concentration determined using a BioRad asaay kit. Dilutions of this extract were tested in a quantitative competitive RIA. Antibody 2A11 was adsorbed to 96-well polyvinylchloride plates at 500 ng per well followed by saturation with 1% BSA-PBS. Unlabelled bombesin (covering a range of 1-1,000 pg per well bombesin for a standard curve) or cell extract peptides at different dilutions were added to triplicate wells and incubated for 1 h at 25 °C. Without removing the unlabelled peptides, 12,000 c.p.m. of ^{125}I-Tyr 4-bombesin (labelled by the chloramine-T methods to 50-80 μCi $μg^{-1}$) was added to each well and incubated overnight at 4 °C, washed eight times and counted. All additions were in 1% BSA-PBS in 0.025-ml volume, and the washes were with 1% BSA-PBS. GRP 1-27, GRP 14-27 and GRP 20-27 were equipotent with bombesin in competing for labelled bombesin and the midpoint of the assay curve detected 25 pg bombesin per well. In contrast, substance P, unrelated peptides and synthetic analogues of bombesin or GRP with substitutions blocking binding to the cellular BLP receptor were unable to significantly compete with labelled bombesin in this assay. In addition, MOPC 21 immunoglobulin did not bind the labelled peptide. Using the standard curve, the BLP equivalent of the cell extract peptides was determined and expressed as pmol of BLP mg^{-1} of acid soluble protein in the extract. Results are the average of triplicate determinations with with variation between replicates of <5%.

mice each cell line produced a palpable tumour mass. The established tumours were then subjected to three experimental treatments (five mice each group) consisting of: (1) phosphate-buffered saline (PBS); (2) the isotype-matched immunoglobulin MOPC 21; and (3) 2A11. Treatment was administered by intraperitoneal (i.p.) injection three times weekly and tumour growth was monitored as a function of tumour volume over the next four weeks of therapy (Fig. 2). The melanoma cell line, NCI-H234A, showed unhampered tumour growth in all three experimental groups with each group of mice attaining an average tumour volume of 1,000 mm^3 by week 5 (Fig. 2*a*). In contrast, dramatic suppression of SCLC growth (NCI-N592) was observed following 2A11 treatment (tumour mass in all mice <50 mm^3) while control groups of MOPC 21- and PBS-treated mice demonstrated rapid tumour growth (tumour mass in all mice >1,000 mm^3 by five weeks) (Figs 2*b*; 3). Note that in MOPC 21-treated controls there was an unexplained temporary delay in tumour growth in some mice, but in all cases a large tumour had formed by five weeks.

In the 2A11-treated group, three more weeks of treatment were given; two mice never showed tumour growth, but three developed progressively growing NCI-N592 tumours despite 2A11 treatment. These antibody-resistant tumours are under study. At week 5, the 5 mice with large NCI-N592 tumours in the PBS-treated group were then treated with 2A11 antibody for three weeks and the tumours in all 5 mice stopped growing and became necrotic.

In conclusion, we have reported on a monoclonal antibody to amphibian bombesin that cross-reacts with BLPs of human origin. The antigenic determinant for this antibody has been localized at the C-terminal region of bombesin and GRP. We have demonstrated that 2A11 can be used in an RIA for the quantitative detection of BLPs in extracts of human SCLC. The antibody was shown to block bombesin-receptor interaction in

Table 3 The effect of monoclonal antibody 2A11 on the *in vitro* clonal growth of human small-cell lung cancer lines

Addition to cloning medium	Cell line NCI-N592 Growth as % control (no of colonies)	NCI-N417 (no of colonies)
Experiment 1 ($\mu g\ ml^{-1}$)		
None	100 (30)	100 (583)
2A11 0.1	93 (28)	98 (571)
2A11 1.0	83 (25)	96 (560)
2A11 3.0	0 (0)	3 (18)
2A11 10	0 (0)	1 (7)
MOPC 21 10	97 (29)	99 (577)
Experiment 2		
None	100 (32)	100 (610)
50 nM bombesin	>100 (664)	>100 (820)
2A11 10 $\mu g\ ml^{-1}$	0 (0)	5 (30)
MOPC 21 10 $\mu g\ ml^{-1}$	94 (30)	100 (607)
Boiled 2A11 10 $\mu g\ ml^{-1}$	88 (28)	97 (593)
2A11 10 $\mu g\ ml^{-1}$ + bombesin 50 nM	>100 (340)	>100 (910)

Tumour cell cloning assays were performed in serum-free HITES medium as described previously[29]. HITES medium contains RPMI-1640 (GIBCO) supplemented with 10 nM hydrocortisone, 5 $\mu g\ ml^{-1}$ bovine insulin, 10 $\mu g\ ml^{-1}$ human transferrin, 10 nM 17,B-estradiol and 30 nM selenium. A single-cell suspension of NCI-N592 or NCI-N417 cells taken from log-phase cultures was mixed with 0.3% agarose in HITES (v/v) in the absence or presence of purified 2A11 or MOPC 21 antibody, bombesin, boiled antibody or 2A11 + bombesin (added simultaneously) and plated over a pre-hardened base layer of 0.5% agarose/HITES. The NCI-N417 culture plates were supplemented with 0.1% BSA which greatly enhanced clonal cell growth. NCI-N592 cells were seeded at 5×10^4 per plate, whereas NCI-N417 cells were plated at 1×10^4. Plates were pre-screened to varify single-cell distribution of test cells and scored 21 days later for colony formation. Cell aggregates of >50 cells were scored positive for colony growth. All studies were done in triplicate and each point represents the mean colony count. For all studies the s.e.m. was ±10%.

both brain and SCLC membrane preparations. Finally, purified antibody when added to an *in vitro* tumour cloning assay or to an *in vivo* nude mouse xenograft assay prevented tumour cell growth. The antibody effect *in vitro* was blocked by synthetic bombesin, and the antibody did not inhibit the growth of a melanoma tumour cell line that did not make BLPs. We conclude that BLPs can indeed function as autocrine growth factor(s) for human SCLC. It is possible that the bombesin stimulation of growth seen in SCLC could reflect a physiological role of BLPs in some cells during normal fetal growth and development. In addition, our studies with monoclonal antibody 2A11 against BLP offer a new immuno-hormonal approach of manipulating the growth of human SCLC in patients. Although these studies used monoclonal anti-peptide antibodies to inhibit BLP action, it is possible that growth suppressive effects could be obtained using bombesin antagonists or anti-receptor antibodies.

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Translocation of the p53 gene in t(15;17) in acute promyelocytic leukaemia

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Recent studies have demonstrated that the cellular tumour antigen p53 (ref. 1) can complement activated *ras* genes in the transformation of rat fibroblasts, suggesting that the gene encoding p53 may act as an oncogene[2,3]. Here, by using *in situ* chromosomal hybridization[4,5], we have mapped the p53 gene to human chromosome 17, at bands 17q21–q22, the region containing one of the breakpoints in the translocation t(15;17) (q22;q21) associated with acute promyelocytic leukaemia (APL)[6,7]. Hybridization of p53 and *erb-A* (17q11–q12) probes to malignant cells from three APL patients indicated that the p53 gene is translocated to chromosome 15 (15q+), whereas *erb-A* remains on chromosome 17. Analysis of variant translocations demonstrates that the 15q+ chromosome contains the conserved junction[8], suggesting a role for p53 in the pathogenesis of APL. However, rearrangements of the p53 gene were not detected on Southern blotting of DNA from leukaemic cells of four APL patients with t(15;17).

To determine the chromosomal localization of the p53 gene, we performed *in situ* chromosomal hybridization using a p53-specific probe. We used an ^{3}H-labelled complementary DNA clone, pp53-176, containing the entire coding region (1.35 kilobases, kb) of the murine p53 gene[9], for *in situ* hybridization to normal human metaphase chromosomes prepared from phytohaemagglutinin-stimulated peripheral blood lymphocytes. Figure 1 shows the distribution of labelled sites in 100 metaphase cells examined. This hybridization resulted in labelling of a specific region of a single chromosome, namely, the long arm of chromosome 17. Of 100 metaphase cells examined, 24 (24%) were labelled on region q2, bands q21–q25, of one or both chromosomes 17. A total of 183 grains was observed; thus, the labelled sites on region q2 of chromosome 17 represented 14.2% of all labelled sites (26/183). The largest number of grains was clustered at 17q21–q22 (18/183, 10%, $P < 0.005$).

The localization of the p53 gene to 17q21–q22 is of particular interest because of the nonrandom abnormalities involving this